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<b>13. ABSTRACT (Maximum 200)</b> To produce human antibody fragments which bind to novel tumor antigens, we have created a very large ( $7.0 \times 10^9$ member) single chain Fv (scFv) phage antibody library from which antibodies can be isolated against any antigen. Methodology is being developed to permit selection of the library on breast tumor cell lines and tissues which will allow isolation of antibodies which bind novel tumor antigens for the purpose of immunotherapy and vaccine development. Methodology has also been developed to increase antibody fragment affinity in vitro. We have determined how to optimally create, select, elute and screen mutant phage antibodies for higher affinity binding. These techniques have been applied to increase the affinity of a human single chain Fv (scFv) antibody fragment (C6.5) which binds to the breast tumor antigen c-erbB-2 1200 fold to a $K_d$ of $1.3 \times 10^{-11}$ M, making it the highest affinity human antibody ever produced to a tumor antigen. Increased affinity results in greater quantitative delivery of scFv to human tumor xenografts. The scFv affinity mutants are being used to construct larger bivalent antibody fragments to permit identification of the optimal size, affinity, and valence for tumor targeting. Using this approach, molecules have been created which have specific tumor targeting values approaching those required for successful radioimmunotherapy.			
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## Table of Contents

Section	Page numbers
1. Introduction	2-5
1.1 Limitations of murine monoclonal antibodies	2
1.2 A new approach to making antibodies	3
1.3 Purpose of the present work and methods of approach	3
2. Body of Report	5-28
3. Conclusions	28-29
4. References	29-34
5. Appendices	
5.1 Appendix 1:	
Schier RS, Marks JD, Wolf EJ, Apell G, Wong C, McCartney JE, Bookman M, Huston J, Houston LL, Weiner LM, and Adams GP. <i>In vitro</i> and <i>in vivo</i> characterization of a human anti-c-erbB-2 single-chain Fv isolated from a filamentous phage antibody library. <i>Immunotechnology</i> . 1: 73-81, 1995.	
5.2 Appendix 2:	
Schier R, Bye J, Apell G, McCall A, Adams GP, Malmqvist M, Weiner LM, Marks JD. Isolation of high affinity human anti-c-erbB-2 single chain Fv using affinity driven selection. <i>J. Mol. Biol.</i> , 255: 28-43, 1996.	
5.4 Appendix 3:	
Schier R and Marks JD. Efficient <i>in vitro</i> affinity maturation of phage antibodies using BIAcore guided selections. <i>Human Antibodies and Hybridomas</i> , In press.	
5.3 Appendix 4:	
Schier R, Balint RF, McCall A, Apell G, Larrick JW, Marks JD. Identification of functional and structural amino acid residues by parsimonious mutagenesis. <i>Gene</i> , 169: 147-155, 1996.	
5.5 Appendix 5:	
Schier R, McCall A, Adams GP, Marshall K, Yim M, Merritt H, Crawford RS, Weiner LM, Marks JD. Isolation of high affinity anti-c-erbB-2 single-chain Fv by molecular evolution of the complementarity determining regions in the center of the antibody combining site. <i>J. Mol. Biol.</i> In press.	
5.6 Appendix 6:	
Schier, RS. & Marks, JD. High affinity human antibodies to novel tumor antigens. Provisional Patent Application.	

## 1. Introduction

A major goal of cancer research has been to identify tumor antigens which are qualitatively or quantitatively different from normal cells (1). The presence of such antigens could be detected by monoclonal antibodies that would form the basis of diagnostic and prognostic tests. In addition, the antibodies could be used to selectively kill tumor cells either directly via their effector function (2) or by attaching cytotoxic molecules to the antibody (3, 4).

Despite the demonstration of antigens which are overexpressed on tumor cells, antibodies have been used with limited success for diagnosis and treatment of solid tumors, (reviewed in ref. (5, 6)). Their utility has been hampered by the paucity of tumor specific antibodies, immunogenicity, low affinity, and poor tumor penetration. For this project, we proposed using a novel technology, termed phage display, to produce a new generation of antibodies which would overcome the limitations of previously produced anti-tumor antibodies. The antibodies would bind breast cancer antigens with high affinity, be entirely human in sequence, and would penetrate tumors better than IgG.

### 1.1 Limitations of murine monoclonal antibodies

Production of monoclonal antibodies from hybridomas requires administration of an immunogenic antigen. Many of the antigens overexpressed on tumor cells are not likely to be immunogenic, since they are also present on normal cells at low levels and would be recognized as 'self antigens'. Thus an immune response would not be generated. In addition, many of the antigens are polysaccharides and do not elicit classic T-cell help needed to trigger the production of higher affinity antibodies. Consequently, many of the antibodies produced are of relatively low affinity. Even when a vigorous immune response is elicited, the affinities ( $K_d$ ) of the resulting monoclonal antibodies are not likely to be better than  $1.0 \times 10^{-9}$  M (7). Finally, it is likely that very few of the antigens overexpressed on tumor cells have been identified, purified and used as immunogens. As an alternative, tumor cells have been used as immunogens in an attempt to elicit an immune response against overexpressed, but as yet unidentified antigens. Instead, antibodies are produced against immunodominant epitopes, but not necessarily against useful tumor antigens.

IgG are also large (150kD) molecules which diffuse slowly into tumors (1 mm every 2 days) (8). The large size of IgG also results in slow clearance from the body and poor tumor:normal organ ratios (9). If the antibody carries a toxic agent, significant bystander damage may result. Recent advances in molecular biology have made it possible to produce (Fab)<sub>2</sub> and Fab in *E. coli*, as well as even smaller single chain Fv molecules (scFv, 25 kD). The scFv consist of the heavy and light chain variable regions (V<sub>H</sub> and V<sub>L</sub>) connected by a flexible peptide linker which retain the binding properties of the IgG from which they were derived (10). Smaller antibody molecules, particularly scFv, are cleared from the blood more rapidly than IgG, and thus provide significantly greater targeting specificity (11). scFv also penetrate tumors much better than IgG in preclinical models (12). The scFv are monomeric, however, and dissociate from tumor antigen significantly faster than divalent IgG molecules, which exhibit a higher apparent affinity due to the avidity effect (13). This feature, combined with rapid clearance from blood, results in significantly lower quantitative retention of scFv in tumor. This limitation could be overcome by creating higher affinity scFv with slower dissociation rate constants or by creating dimeric scFv (11).

A final disadvantage of murine antibodies or antibody fragments is that they are likely to be immunogenic when administered therapeutically. Murine or chimaeric IgG are clearly immunogenic when administered to humans and some of the immune response is directed against the variable regions (14). The smaller size antibody fragments should be less immunogenic, but this still may be a problem when repeated doses are required for therapy. Thus therapeutic antibodies would ideally be of human origin. Unfortunately, production of human antibodies using hybridoma technology has proven extremely difficult, especially antibodies against human proteins such as tumor antigens (15).

All of the above limitations can be overcome by taking advantage of recent advances in biotechnology to produce human antibody fragments directly in bacteria without immunization (reviewed in (16, 17). Bacterial libraries containing billions of human antibody fragments are created, from which binding antibody fragments (scFv or Fab) can be selected by antigen. This approach will overcome the limitations of conventional hybridoma technology discussed above. Immunization is not required, purified antigen is not necessary, and it will be possible to isolate antibodies to overexpressed 'self' antigens which would not be immunogenic *in vivo*. The affinities of the antibody fragments would be increased *in vitro*, to values not achievable using conventional hybridoma technology. The result would be production of unique tumor specific monoclonal antibodies with binding properties not previously available.

## 1.2 A new approach to making antibodies

The ability to express antibody fragments on the surface of viruses which infect bacteria (bacteriophage or phage) makes it possible to isolate a single binding antibody fragment from a library of greater than  $10^{10}$  nonbinding clones. To express antibody fragments on the surface of phage (phage display), an antibody fragment gene is inserted into the gene encoding a phage surface protein (pIII) and the antibody fragment-pIII fusion protein is displayed on the phage surface (18, 19). Since the antibody fragments on the surface of the phage are functional, phage bearing antigen binding antibody fragments can be separated from non-binding phage by antigen affinity chromatography (18). Depending on the affinity of the antibody fragment, enrichment factors of 20 fold - 1,000,000 fold are obtained for a single round of affinity selection. By infecting bacteria with the eluted phage, however, more phage can be grown and subjected to another round of selection. In this way, an enrichment of 1000 fold in one round can become 1,000,000 fold in two rounds of selection (18). Thus even when enrichments are low (20), multiple rounds of affinity selection can lead to the isolation of rare phage. Since selection of the phage antibody library on antigen results in enrichment, the majority of clones bind antigen after 4 rounds of selection. Thus only a relatively small number of clones (several hundred) need to be analyzed for binding to antigen. Analysis for binding is simplified by including an amber codon between the antibody fragment gene and gene III. The amber codon makes it possible to easily switch between displayed and soluble (native) antibody fragment simply by changing the host bacterial strain (19).

Human antibodies can be produced without prior immunization by displaying very large and diverse V-gene repertoires on phage (20). In the first example, natural  $V_H$  and  $V_L$  repertoires present in human peripheral blood lymphocytes were isolated from unimmunized donors by PCR. The V-gene repertoires were spliced together at random using PCR to create a scFv gene repertoire which was cloned into a phage vector to create a library of 30 million phage antibodies (20). From this single "naive" phage antibody library, binding antibody fragments have been isolated against more than 17 different antigens, including haptens, polysaccharides and proteins (20-22). Antibodies have been produced against self proteins, including human thyroglobulin, immunoglobulin, tumor necrosis factor and CEA (22). It is also possible to isolate antibodies against cell surface antigens by selecting directly on intact cells. For example, antibody fragments against 4 different erythrocyte cell surface antigens were produced by selecting directly on erythrocytes (21). Antibodies were produced against blood group antigens with surface densities as low as 5,000 sites/cell. The antibody fragments were highly specific to the antigen used for selection, and were functional in agglutination and immunofluorescence assays. Antibodies against the lower density antigens were produced by first selecting the phage antibody library on a highly related cell type which lacked the antigen of interest. This negative selection removed binders against the higher density antigens and subsequent selection of the depleted phage antibody library on cells expressing the antigen of interest resulted in isolation of antibodies against that antigen. With a library of this size and diversity, at least one to several binders can be isolated against a protein antigen 70% of the time (J.D. Marks, unpublished data). The antibody fragments are highly specific for the antigen used for selection and have affinities in the 1  $\mu$ M to 100 nM range (20, 22). Larger phage antibody

libraries result in the isolation of more antibodies of higher binding affinity to a greater proportion of antigens.

Phage display is also an effective technique for increasing antibody affinity. Mutant scFv gene repertoires, based on the sequence of a binding scFv, are created and expressed on the surface of phage. Higher affinity scFv are selected by affinity chromatography on antigen as described above. One approach for creating mutant scFv gene repertoires has been to replace the original V<sub>H</sub> or V<sub>L</sub> chain with a repertoire of V-genes to create new partners (chain shuffling) (23). Using chain shuffling and phage display, the affinity of a human scFv antibody fragment which bound the hapten phenylloxazalone (phOx) was increased from 300 nM to 1 nM (300 fold) (24).

### 1.3 Purpose of the present work and methods of approach

For this work, we proposed to isolate and characterize a large assortment of high affinity human antibody fragments that bound to specific breast cancer antigens and to normal antigens that are overexpressed on cancer cells. Human antibodies isolated using phage display would be used for early sensitive diagnosis of node-negative breast cancer patients, for immunotherapy prior to growth of large tumor mass, and as adjuvant therapy for minimal residual disease. Human antibody repertoires were to be created from the mRNA of healthy individuals using the polymerase chain reaction, and cloned to create a very large and diverse phage antibody library of >10,000,000,000 different members. This phage antibody library would be at least 300 times larger than previous libraries, and hence would contain a greater number of antibodies against more epitopes on more antigens. The affinities of the initial isolates would also be higher. Antibodies that recognize antigens which are overexpressed or unique to breast carcinomas would be isolated by selection on breast tumor antigens or cell lines and characterized with respect to affinity and specificity. Affinities were to be increased by mutagenesis of the antibody genes, construction of mutant phage antibody libraries, and selection on tumor cells.

The proposed technical objectives were:

- 1.3.1 Isolate human scFv antibody fragments which bind breast tumor antigens using a pre-existing scFv phage antibody library.
- 1.3.2 Create a non-immune human Fab phage antibody library containing  $10^9$ - $10^{11}$  members.
- 1.3.3 Isolate human Fab antibody fragments which bind breast tumor antigens by selecting this new non-immune Fab phage antibody library on primary and metastatic breast tumor cell lines.
- 1.3.4 Characterize binding scFv and Fabs with respect to DNA sequence, specificity, and affinity.
- 1.3.5 Increase the affinity of antibody fragments with the desired binding characteristics by creating mutant phage antibody libraries and selecting on the appropriate breast tumor cell line.
- 1.3.6 Characterize mutant antibody fragments with respect to DNA sequence, specificity, and affinity.

In the Statement of Work, we estimated that during the two years of this 4 year grant, we would create a large Fab phage antibody library and screen the phage antibody libraries on breast tumor cell lines.

## 2. Body of report

The work in the second year of the grant builds directly on results produced during the first year of funding. In addition, some of the results in last years report were either preliminary or only partially complete. For both of these reasons, I have included some results

previously reported in the year 1 report. To facilitate distinction, each section is broken down by year, although this distinction is somewhat arbitrary.

Work during the first two years of the grant focused on creation of a large phage antibody library (technical objective 1.3.2), and screening of the scFv phage antibody libraries (technical objective 1.3.1) on breast tumor cell lines. Work was also begun to identify the optimal means of increasing antibody fragment affinity (technical objective 1.3.5) using a human scFv (C6.5) isolated from a non-immune phage antibody library (25). C6.5 binds the breast tumor antigen c-erbB-2. As a result of this work, we have been able to develop an efficient and effective approach to create, identify, and characterize higher affinity antibody fragments in vitro. Using this approach, we have engineered the affinity of C6.5 to produce mutants with affinities between  $1.0 \times 10^{-6}$  M to  $1.3 \times 10^{-11}$  M. The best binders represent the highest affinity antibodies ever produced to any tumor antigen and our results provide a general approach to rapidly increase antibody affinity to values not achievable with animal immunization. The antibody fragments also permit for the first time examination of the relationship between affinity and specific tumor targeting using antibodies that differ by only a few amino acids in sequence and which recognize identical epitopes. During the most recent funding year, c-erbB-2 targeting single chain Fv have been examined in *scid* mice bearing human SK-OV-3 tumors. Over the range of affinities examined in detail to date ( $K_d = 3.2 \times 10^{-7}$  to  $1.0 \times 10^{-9}$  M) increased affinity correlates with greater quantitative retention of scFv in tumor. Preliminary targeting results with higher affinity scFv ( $K_d = 1.6 \times 10^{-10}$  to  $1.3 \times 10^{-11}$  M) are also reported. We have also begun to examine the effect of size and valency on targeting using the monomeric scFv as building blocks to create larger multivalent molecules. A dimeric diabody (scFv)<sub>2</sub> has been constructed from the genes of C6.5 and its in vitro cell retention and in vivo targeting are reported.

## 2.1 Creation of a large scFv phage antibody library

### Year 1

In the original grant application, we had proposed creating a large Fab phage antibody library using combinatorial infection. By the time work was begun on the project, a large Fab phage antibody library ( $7.0 \times 10^{10}$  members) had already been created in the Laboratory of Dr. Greg Winter, using combinatorial infection (26). In the initial publication, this library was an excellent source for obtaining high affinity antibodies to small molecules (haptens) but only a relatively few Fabs with affinities ( $K_d$ ) between  $5.0 \times 10^{-8}$  to  $1.0 \times 10^{-8}$  M were isolated against protein antigens (26). This library was kindly made available to us for use in this project by Dr. Greg Winter. Manipulation of the library revealed 2 major limitations: 1) expression levels of Fabs was too low to produce adequate material for characterization, and 2) the library was relatively unstable. These limitations are a result of creating the library in a phage vector, and the use of the cre-lox recombination system. We therefore decided that the best approach for this project was to create a very large scFv library using a phagemid vector. The goal was to produce a library at least 100 times larger than our previous  $3.0 \times 10^7$  member scFv library. The approach taken was to clone the  $V_H$  and  $V_L$  library on separate replicons, combine them into an scFv gene repertoire by splicing by overlap extension, and clone the scFv gene repertoire into the phage display vector pHEN1 (19). Human peripheral blood lymphocyte and spleen RNA was primed with immunoglobulin  $C_{\kappa}$ ,  $C_{\lambda}$ , and IgM primers, and 1st strand cDNA synthesized. 1st strand cDNA was used as a template for PCR amplification of the  $V_H$ ,  $V_{\kappa}$ , and  $V_{\lambda}$  gene repertoires. The  $V_H$  gene repertoires were cloned into the vector pUC119Sfi-Not as Nco1-NotI fragments, to create a library of  $3.2 \times 10^8$  members. The library was diverse by PCR fingerprinting. Single chain linker DNA was spliced onto the  $V_{\kappa}$  and  $V_{\lambda}$  gene repertoires using PCR and the repertoire cloned as an XhoI-NotI fragment into the vector pHENIXscFv to create a library of  $1.6 \times 10^6$  members. The  $V_H$  and  $V_L$  gene repertoires were amplified from their respective vectors and spliced together using PCR to create an scFv gene repertoire. The scFv

gene repertoire was cloned as an NcoI-NotI fragment into the vector to create an scFv phage antibody library of  $7.0 \times 10^9$  members. The library was diverse as determined by BstNI fingerprinting.

### Year 2

To verify the quality of the library, phage were prepared and selected on 8 different antigens. The results are shown in Table 1. scFv antibodies were obtained against all antigens used for selection, with between 8 and 15 unique scFv isolated per antigen (Table 1). This compares favorably to results obtained from smaller scFv libraries (1 to a few binders obtained against only 70% of antigens used for selection). Affinities of 8 anti-erbB-2 scFv were measured using surface plasmon resonance in a BIAcore and found to range from  $2.5 \times 10^{-8}$  M to  $3.2 \times 10^{-10}$  M. Thus the library was established as a source of panels of human antibodies against any antigen with affinities at least equivalent to the secondary murine response.

**Table 1. Specificities and number of binders isolated from a  $7.0 \times 10^9$  member scFv phage antibody library.**

Antigen	Number of unique binders
prostate specific antigen	12
vascular endothelial growth factor	12
bone morphogenic protein receptor	9
activin receptor type 1	11
activin receptor type 2	9
cytochrome b5	8
erbB-2	10
fibroblast growth factor receptor	11
$\alpha$ -bungarotoxin	15

## 2.2A Selection of the smaller scFv phage antibody library on breast tumor cell lines

### Year 1

A  $3.0 \times 10^7$  member scFv phage antibody library was selected on the malignant breast tumor cell lines MB231 and ZR-75-1, both with and without negative selections on the normal breast cell line HBL100. Results from both types of selections resulted in the isolation of scFv that bound both malignant and non-malignant cell lines. Antibodies from this library are known to be of low affinity, and this results in poor depletion of scFv that bind antigens common to malignant and normal cell lines. The low affinities also result in low enrichment ratios on the relevant cell type. Rather than spend time optimizing selections using this library, we focused on production of a much larger scFv phage antibody library. Larger libraries will contain a greater number of high affinity binders, resulting in more effective depletion of scFv that bind antigens in common, and greater positive enrichment ratios.

### 2.2B Selection of the large scFv phage antibody library on breast tumor cell lines

The  $7.0 \times 10^{10}$  member scFv phage antibody library described in section 2.1 was selected on the malignant breast tumor cell lines MB231 and ZR-75-1, both with and without negative selections on the normal breast cell line HBL100. Similar results were obtained as described in section 2.2A above. scFv were isolated that could not distinguish malignant from non-malignant cell lines.

#### Significance:

The selection techniques utilized did not permit the isolation of scFv to antigens unique to malignant cell lines, even with the use of negative selection. Thus efforts will be directed to: 1) optimization of selection techniques; and 2) creation of libraries enriched for binders to breast tumor antigens. To optimize selection techniques, we will use c-erbB-2 binding phage antibodies (see below) as a model for cell surface selections. Phage libraries will be 'spiked' with low concentrations of C6.5 based c-erbB-2 binding scFv, and a variety of depletion and

enrichment schemes utilized employing c-erbB-2 positive and c-erbB-2 negative cell lines. This will permit determination of the optimal technique for cell surface selection of phage antibodies. Two approaches will be used to increase the likelihood of producing antibody fragments that bind to novel and relevant breast tumor antigens. First, phage antibody libraries enriched for antibodies binding breast tumor antigens will be created by immunizing mice with two different breast tumor cell lines (MDA MB231 and ZR-75-1). In a separate set of experiments, a subtractive immunization technique will be used to deplete the murine repertoire of antibodies binding antigens which are not tumor specific. This will be accomplished by first immunizing mice with the normal breast cell line HBL 100, followed by administration of cyclophosphamide to kill off B-cells stimulated to proliferate in response to normal cell antigens. After the drug is allowed to clear, the mice will be immunized with the tumor cell line. Splens will be harvested and used to prepare phage antibody libraries.

### **2.3. Optimization of techniques for increasing antibody affinity in vitro, and application to produce ultra-high affinity human antibody fragments which bind the breast tumor antigen c-erbB-2**

Phage display has the potential to produce antibodies with affinities that cannot be produced using conventional hybridoma technology (24, 27-31). Ultra high affinity human antibody fragments could result in excellent tumor penetration, prolonged tumor retention, and rapid clearance from the circulation, leading to high specificity. We therefore undertook a series of experiments to produce ultra high affinity human antibody fragments. During the initial years, experiments were performed to answer the following questions: 1) What is the most effective way to select and screen for rare higher affinity phage antibodies amidst a background of lower affinity binders; 2) What is the most effective means to remove bound phage from antigen, to ensure selection of the highest affinity phage antibodies; 3) What is the most efficient techniques for making mutant phage antibody libraries (random mutagenesis or site directed mutagenesis; 4) What region of the antibody molecule should be selected for mutagenesis to most efficiently increase antibody fragment affinity.

To answer these questions, we studied the human scFv C6.5, which binds the extracellular domain (ECD) of the tumor antigen c-erbB-2 (32) with a  $K_d$  of  $1.6 \times 10^{-8}$  M and  $k_{off}$  of  $6.3 \times 10^{-3} \text{ s}^{-1}$  (25). Isolation and characterization of C6.5 is described briefly below and in detail in Schier et al., appendix 1. The isolation and initial characterization of C6.5 was partially supported by this grant, as well as by a subcontract to the Marks lab by National Cooperative Drug Discovery Group Group Award U01 CA 51880. Results of the experiments described below provide a general approach for efficiently increasing antibody affinity and yielded human antibody fragments to the breast tumor antigen c-erbB-2 with up to an 1200 fold increased affinity ( $K_d = 1.3 \times 10^{-11}$  M). This represents the highest affinity tumor targeting antibody produced by any means. In subsequent sections, we report the effects of antibody fragment affinity, valence, and size on in vitro tumor cell binding and in vivo tumor targeting.

#### **Year 1**

##### **2.3.1. Isolation and characterization of C6.5, a human scFv which binds c-erbB-2 ECD**

Human scFv which bound to c-erbB-2 ECD were isolated by selecting the non-immune human scFv phage antibody library (described in section 2.2 above) on c-erbB-2 extracellular domain immobilized on polystyrene. After five rounds of selection, 45/96 clones analyzed produced scFv which bound c-erbB-2 by ELISA. Restriction fragments analysis and DNA sequencing revealed the presence of two unique human scFv, C4 and C6.5. Both of these scFv bound only to c-erbB-2 and not to a panel of 10 irrelevant antigens. Cell binding assays, however, indicated that only C6.5 bound c-erbB-2 expressed on cells, and thus this scFv was selected for further characterization.

##### **2.3.1A. Method for purification of C6.5**

To facilitate purification, the C6.5 scFv gene was subcloned into the expression vector pUC119 Sfi-NotmycHis which results in the addition of the myc peptide tag followed by a hexahistidine tag at the C-terminal end of the scFv. The vector also encodes the pectate lyase leader sequence which directs expression of the scFv into the bacterial periplasm where the leader sequence is cleaved. This makes it possible to harvest native properly folded scFv directly from the bacterial periplasm, without the need for refolding. Native C6.5 scFv was expressed (33) and purified from the bacterial supernatant using immobilized metal affinity chromatography (IMAC) (34). The yield after IMAC purification and gel filtration on a Superdex 75 column was 10.5 mg/L.

**Significance:**

This vector and purification scheme provide a generic technique for rapid two step scFv purification. This permits us to quickly purify many different mutant scFv in high yield for further in vitro and in vivo characterization. To date, we have purified more than 150 different scFv using this technique (see below and manuscripts in appendix 2, 3, 4 and 5, and the patent application in appendix 6 for examples).

**2.3.1B. Method for measurement of C6.5 affinity for c-erbB-2**

The  $K_d$  of C6.5 and the kinetics of binding to c-erbB-2 were determined in a BIAcore, a biosensor based on surface plasmon resonance (35). For this technique, antigen is coupled to a derivatized sensor chip capable of detecting changes in mass. When antibody is passed over the sensor chip, antibody binds to the antigen resulting in an increase in mass which can be quantitated. Measurement of the rate of association as a function of antibody concentration can be used to calculate the association rate constant ( $k_{on}$ ). After the association phase, buffer is passed over the chip and the rate of dissociation of antibody ( $k_{off}$ ) can be determined.  $k_{on}$  can be measured in the range  $1.0 \times 10^2$  to  $5.0 \times 10^6$  and  $k_{off}$  in the range  $1.0 \times 10^{-1}$  to  $1.0 \times 10^{-6}$ . The equilibrium constant  $K_d$  can be calculated as  $k_{off}/k_{on}$  and thus can be measured in the range  $10^{-5}$  to  $10^{-12}$ . Affinities and dissociation rate constants measured by surface plasmon resonance correlate closely to values determined on cells expressing c-erbB-2 (see below).

**2.3.1C. Affinity of C6.5 for c-erbB-2**

The kinetics of binding and affinity of purified C6.5 were determined by BIAcore and the results are shown in Table 2 (see also Schier et al, appendix 1). The  $K_d$  of  $1.6 \times 10^{-8}$  M determined by BIAcore is in close agreement to the  $K_d$  determined by Scatchard analysis after radioiodination ( $2.0 \times 10^{-8}$  M). Biodistribution of C6.5 was determined and the % injected dose/gm tumor at 24 hours was 1.1% with tumor/organ ratios of 5.6 for kidney and 103 for bone.

**Significance:**

1. A rapid technique based on the BIAcore was developed to measure affinity of scFv for the tumor antigen c-erbB-2. Affinities measured using this technique correlate well with affinities measured by Scatchard after radioiodination. In addition, the technique does not require any labeling of the scFv.
2. A human scFv which binds specifically with moderate affinity to c-erbB-2 as expressed on tumor cells has been produced. The scFv expresses at high level in *E. coli* as native scFv and can be easily purified in high yield in two steps. This scFv was used as a model for development and optimization of techniques for increasing antibody fragment affinity in vitro.

**2.3.2. Optimization of conditions for selecting higher affinity monomeric scFv (Schier et al., 1996, appendix 2)**

Successful selection for higher affinity antibody fragments requires optimization of antigen presentation and antigen concentration. Typically, antigen has been immobilized on a

solid phase (e.g. polystyrene tubes or microtitre plates). Alternatively, non-limiting concentrations of biotinylated antigen have been used in solution, followed by capture of bound phage using streptavidin coated paramagnetic beads. To determine the optimal method of antigen presentation for selecting higher affinity scFv, we selected a mutant C6.5 phage antibody library on c-erbB-2 immobilized on polystyrene tubes, or on biotinylated c-erbB-2 in solution. Isolation of higher affinity scFv was dependent on the selection conditions used (experimental conditions and results are described in detail in Schier et al., appendix 2). When selections were performed on antigen immobilized on polystyrene, scFv were isolated which existed in solution as mixtures of monomer and dimer (see figure 2, appendix 2). Dimerization and oligomerization have been observed with other scFv (21, 22, 36-42), and result from the V<sub>H</sub> domain of one scFv molecule pairing with the V<sub>L</sub> domain of a second scFv molecule, and vice versa (37, 41). The resulting homodimeric scFv have two binding sites which can result in a significant increase in apparent affinity (avidity) when binding to multivalent antigen (22, 37, 39, 41, 42). The tendency of scFv to dimerize is sequence dependent, with some scFv existing as stable monomer (22, 25, 37, 38), and others as mixtures of monomeric and oligomeric scFv (22, 38, 40-42). Thus, a phage antibody library will consist of some phage with monomeric scFv on the surface, and other phage with dimeric scFv on the surface. Dimeric scFv can form on the phage surface by noncovalent association of the V-domains of the scFv-pIII fusion with the V-domains of native scFv in the periplasm. Native scFv appears in the periplasm both from incomplete suppression of the amber codon between the scFv gene and gene III, as well as by proteolysis. Our results demonstrate that dimeric scFv will be selected preferentially over monomeric scFv when selections are performed on immobilized antigen, due to avidity (see Table 2 and figure 2, appendix 2). This selection bias interferes with the selection of scFv with truly higher monovalent affinity and may explain the failure of Deng et al. to isolate higher affinity anti-carbohydrate scFv from a phage display library selected on multivalent antigen immobilized on polystyrene (42). Instead scFv with a greater tendency to dimerize were isolated. Our results also indicate that a relatively small number of amino acid substitutions (7 or less) can convert a monomeric scFv to an scFv forming mixtures of monomer and dimer (see Table 3 and figure 2, appendix 2).

Experimental results suggest scFv dimerization depends on the tendency of V<sub>H</sub> and V<sub>L</sub> domains to dissociate (41). As measured on Fv fragments, the V<sub>H</sub>-V<sub>L</sub> dissociation constant is typically high ( $10^{-6}$  M), but can differ at least 100 fold between different Fv ( $10^{-6}$  M to  $10^{-8}$  M) (43-45). When the dissociation constant is high, the V<sub>H</sub> and V<sub>L</sub> domains on the same scFv dissociate and pair with domains on another scFv molecule. Differences in the V<sub>H</sub>-V<sub>L</sub> K<sub>d</sub> result from differences in residues composing the  $\beta$ -sheets which make up the V<sub>H</sub>-V<sub>L</sub> interface (46). While many of these interface residues are conserved, 25% of the interface results from residues in the hypervariable CDRs (46). Interestingly, three of the 4 mutants which dimerize have substitutions in amino acids which comprise at least one of the  $\beta$ -strands in the interface (Table 3 and figure 3, appendix 2). The fourth has an insertion in one of the interface  $\beta$ -strands. In 3 of these scFv, the mutations occur in V<sub>L</sub> CDR3. The effect of these mutations may be to reduce V<sub>H</sub>-V<sub>L</sub> affinity, resulting in dissociation and subsequent dimer formation.

Isolation of higher affinity monomeric scFv resulted from selections performed in solution on biotinylated antigen with subsequent capture on streptavidin magnetic beads (Tables 2 and 4, and figure 2, appendix 2). Selecting in solution reduces the avidity effect of dimeric scFv. For the initial rounds of selection, an antigen concentration greater than the K<sub>d</sub> of the wild type scFv was used in order to capture rare, or poorly expressed, phage antibodies (Table 2, appendix 2). To select on the basis of affinity, an antigen concentration significantly less than the desired K<sub>d</sub>, and less than the phage concentration, was used in the latter rounds of selection (Table 2, appendix 2). In the case of V<sub>L</sub> shuffling, higher affinity binders were obtained with either of the antigen concentration regimens used, but the greatest enrichment for higher affinity binders was obtained at the lowest antigen concentration ( $1.0 \times 10^{-11}$  M) (Table 2,

appendix 2). In the case of  $V_H$  shuffling, higher affinity binders were only obtained at the lowest antigen concentration ( $1.0 \times 10^{-11}$  M) (Table 2, appendix 2). Thus the greatest enrichment for higher affinity binders was obtained by limiting the antigen concentration to less than the phage concentration (typically  $10^{-8}$  M) and the desired  $K_d$ . Alternatively, non-limiting antigen concentration has been used to select three fold higher affinity lysozyme binding scFv from a phage antibody library. In this case, however, a phage vector was used and thirteen rounds of selection were utilized (28), suggesting that selections using non-limiting antigen concentration are not as stringent. It is not possible to use thirteen rounds of selection with a phagemid vector, since mutants with deleted antibody genes accumulate and take over the library (J.D. Marks, unpublished data). We prefer the use of a phagemid vector, due to its higher transformation efficiency and ability to easily produce native scFv.

#### Significance:

1. scFv exhibit sequence dependent dimerization and oligomerization that can result in higher apparent affinity due to avidity.
2. Selection of higher affinity monomeric scFv requires that selections be performed in solution using biotinylated antigen to prevent the selection of lower affinity scFv which form mixtures of monomeric, dimeric, and higher molecular weight scFv.
3. Optimal selections result from the use of limiting antigen, using a concentration less than the desired  $K_d$ .

#### **2.3.3. Development of a technique for monitoring stringency of selections (Schier et al., in press, appendix 3)**

As described in section 2.3.2 above, for selection on the basis of affinity, an antigen concentration significantly less than the desired  $K_d$  must be used. Thus the goal is to reduce the antigen concentration to the lowest concentration that results in positive selection. If the concentration is too high, then more lower affinity binders are selected (see Table 2, appendix 2). If the concentration is reduced too low, then few specific phage antibodies will be selected, and deletion mutants will take over. The antigen concentration that should be used for selection can vary significantly, and depends on the expression level of different phage antibodies, and on the different  $K_d$  for antigen.

We have determined experimentally that it is possible to determine if the proper antigen concentration is being used by monitoring the selection process using SPR in a BIAcore. Phage are prepared after each round of selection, and analyzed for binding to c-erbB-2 using SPR in a BIAcore. Due to the size of phage particles, and their relatively low maximal concentration ( $10^{12}$  to  $10^{13}$  particles/ml), the association phase of phage antibody binding to antigen is mass transport limited. Thus the rate of binding is proportional to the concentration of binding phage. In fact, either the change in resonance units (RU) of phage bound/minute or the amount (RU) bound correlated linearly with the log of the phage concentration (figure 1, appendix 3).

This provides a technique for determining the concentration of binding phage in a polyclonal population after each round of selection. Results of such a monitoring process can be seen in Table 1 appendix 3. A C6.5 based mutant phage antibody library was created and selected on decreasing concentrations of biotinylated c-erbB-2 ECD in solution. The titre of eluted phage decreased the first four rounds of selection but the RU of bound phage went up. This indicates positive (and stringent) selection. When the antigen concentration was reduced too low (round 5), the titre of eluted phage went up (due to overgrowth of deletion mutants) and the amount of bound phage decreased. Moreover, the percent positive clones as determined by ELISA correlated well with the percent of binding phage (ratio of binding phage concentration determined by BIAcore and titre of phage preparation applied to the sensor surface (Table 4, appendix 3). Thus by monitoring polyclonal phage preparations after each

round of selection, we can determine if the selection is too stringent, and after which rounds of selection to do ELISA assays.

Significance:

The stringency of phage antibody selections can be monitored using a BIAcore by analyzing polyclonal phage prepared after each round of selection. This permits the use of the lowest possible antigen concentration for each round of selection. This allows the greatest discrimination with respect to affinity without the overgrowth of deletion mutants.

**2.3.4. Development of a technique for estimating affinity of unpurified scFv for antigen (c-erbB-2) (Schier et al., 1996, appendix 2)**

Relative apparent enrichment ratios of phage antibodies are not only dependent on affinity, but are also affected by factors such as scFv expression level, folding efficiency, and level of toxicity to *E. coli*. Thus, the affinity of selected scFv will vary considerably (29), and a technique is needed to identify which of the selected clones are of higher affinity, without having to subclone, sequence, and purify each mutant. A technique frequently used by others to rank mutant antibody fragments is competition ELISA (47). This technique was used to screen mutant scFv created by chain shuffling (see below), however no correlation was found between IC<sub>50</sub> determined by competition ELISA on unpurified scFv in bacterial periplasm and scFv affinity determined on purified protein using surface plasmon resonance in a BIAcore. We therefore developed a screening technique using the BIAcore. Since increased affinity results primarily from a reduction in the k<sub>off</sub>, measurement of k<sub>off</sub> should identify higher affinity scFv. k<sub>off</sub> can be measured in the BIAcore on unpurified scFv in bacterial periplasm, since expression levels are high enough to give an adequate binding signal and k<sub>off</sub> is independent of concentration. The value of k<sub>off</sub> for periplasmic and purified scFv is in close agreement (Table 2).

Table 2. Comparison of k <sub>off</sub> determined on scFv in bacterial periplasm and after purification by IMAC and gel filtration.	scFv	k <sub>off</sub> (s <sup>-1</sup> )
	C6-5 periplasm	5.7 x 10 <sup>-3</sup>
	C6-5 purified	6.3 x 10 <sup>-3</sup>
	C6-5ala3 periplasm	9.3 x 10 <sup>-3</sup>
	C6-5ala3 purified	1.5 x 10 <sup>-2</sup>
	C6-5ala10 periplasm	3.7 x 10 <sup>-3</sup>
	C6-5ala10 purified	4.1 x 10 <sup>-3</sup>

Significance:

A technique has been developed which allows ranking of mutant scFv by k<sub>off</sub>, and hence relative affinity, without purification. This significantly increases the rate at which mutant scFv can be characterized, and markedly reduces the number of mutant scFv subcloned and purified which do not show better binding characteristics than wild type (see results of light chain shuffling and V<sub>L</sub> and V<sub>H</sub>CDR3 randomization below).

**2.3.5. Development of a technique to determine optimal elution conditions to use during phage antibody selections (Schier et al., in press, appendix 3)**

During the selection process, phage antibodies are allowed to bind to biotinylated antigen, the antigen is captured on streptavidin coated magnetic beads, the beads are washed, and specifically bound phage eluted. For selection of the highest affinity antibodies, it is necessary to ensure that all specifically bound phage are eluted. Solutions used for elution include soluble antigen (23, 27, 29), 100 mM triethylamine (20, 22, 24, 26, 27, 29), glycine, pH 2.2 (48), 100 mM NaOAc, pH 2.8 containing 500 mM NaCl (31), or 76 mM citric acid, pH 2.8 (21). Alternatively, magnetic beads with bound phage can be added directly to *E. coli*, resulting in infection rates that are the same as after elution (49).

During affinity maturation of (C6.5), we suspected that elution conditions might not be optimal for eluting the highest affinity binders. To determine if differences existed between elution solutions, we studied an scFv phage antibody library consisting of C6.5 mutants where the CDR3 of the light chain was partially randomized at 9 amino acid positions (see below). This library contained C6.5 mutant scFv with affinities up to 16 times higher than C6.5. Phage were prepared after the third round of selection, and allowed to bind to a c-erbB-2 coated CM5 sensor chip in a BIAcore. The efficacy of 5 different elution solutions was determined by passing the solution over the sensor chip surface and determining the amount of phage that remained bound. Significant differences existed between solutions (Table 2, appendix 3). The most effective solutions were 50 mM and 100 mM HCl. 2.6 M MgCl<sub>2</sub>, which would remove 100% of wild type C6.5, removed only 23% of the polyclonal C6.5 mutants.

To verify that differences observed in elution conditions on the BIAcore were reflected in the affinities of scFv selected, a fourth round of selection was performed on biotinylated c-erbB-2, and the phage eluted with one of 7 eluents: 1) 100 mM HCl, pH 1.0; 2) 50 mM HCl, pH 1.5; 3) 10 mM HCl, pH 2.0; 4) 2.6 M MgCl<sub>2</sub>; 5) 100 mM triethylamine, pH 11.5; 6) 1 μM c-erbB-2 ECD; 7) No elution (magnetic beads resuspended in 1 ml of PBS). The highest affinity scFv from each of the elutions were identified by measuring the  $k_{off}$  on unpurified scFv in bacterial periplasm of 20 ELISA positive clones. The eight highest affinity scFv identified by  $k_{off}$  screening were subcloned, purified,  $k_{on}$ ,  $k_{off}$ , and  $K_d$  determined, and the DNA sequenced. Significant differences were observed in the  $K_d$  of the selected scFv, depending on which elution solution was used (Table 3 and 4, appendix 3). The highest affinity scFv were obtained when eluting with solutions demonstrated by BIAcore to be most efficacious in removing bound phage (100 mM HCl).

#### Significance:

Significant differences exist between solutions frequently used to elute bound phage with respect to the affinities of selected phage antibodies. The optimal elution solution is likely to vary with the particular epitope and range of affinities present in the library. The optimal elution solution can be predicted by BIAcore analysis of a polyclonal phage preparation. Appreciation of these facts and determination of the optimal solution for elution should result in more efficient selection of higher affinity phage antibodies.

#### **2.3.6 Increasing the affinity of C6.5 by random mutagenesis (chain shuffling) (Schier et al., 1996, appendix 2)**

When designing a mutant phage antibody library, decisions must be made as to how and where to introduce mutations. The process of somatic hypermutation can be mimicked by introducing mutations randomly, for example by chain shuffling (23, 50), error prone PCR (27), or the use of mutator strains (51). In the experiments described below, chain shuffling (23, 50) was used to randomly introduce mutations into the V<sub>H</sub> and V<sub>L</sub> genes of C6.5. This method relies on the natural diversity of V-genes present normally in the human repertoire. Chain shuffling has been successfully used to increase the affinity of a non-immune human scFv which bound the hapten phenyloxazolone 300 fold from  $3.0 \times 10^{-7}$  M to  $1.0 \times 10^{-9}$  M by sequentially shuffling the rearranged V<sub>L</sub> gene and the V<sub>H</sub> gene segment (the wild type V<sub>H</sub>CDR3 was retained) (24). Most relevant antigens, however, are proteins, and it is unclear whether chain shuffling would be effective to increase the affinity of protein binding antibody fragments. Shuffling immune rearranged V<sub>H</sub> and V<sub>L</sub> genes of gp120 binding Fabs resulted in Fabs of "similar apparent binding constants" (52, 53). Compared to antibodies which bind haptens, there are a greater number of contacts between protein and antibody with a greater surface area buried upon binding. Thus the chances of disrupting multiple favorable contacts by shuffling is greater, but could be compensated by the loss of unfavorable contacts, or generation of new contacts. For this work, we investigated the utility of chain shuffling to increase the affinity of C6.5. Universal phage display vectors were created which contained

either a human V<sub>H</sub> gene segment repertoire or a rearranged V<sub>L</sub> gene repertoire. These vectors permit light chain shuffling by subcloning the rearranged V<sub>H</sub> gene from an antigen binding scFv, and heavy chain shuffling by subcloning the rearranged V<sub>L</sub> gene, linker, and V<sub>H</sub> CDR3. The shuffling vectors were used to increase the affinity of C6.5 for c-erbB-2 6 fold to  $2.5 \times 10^{-9}$  M, comparable to the affinity of antibodies to the same antigen produced from hybridomas. The work is described briefly below, and in detail in Schier et al., 1996, appendix 2.

To alter the affinity of C6.5, a mutant scFv gene repertoire was created containing the V<sub>H</sub> gene of C6.5 and a human V<sub>L</sub> gene repertoire (light chain shuffling). The scFv gene repertoire was cloned into the phage display vector pHEN-1 and after transformation a library of  $2 \times 10^5$  transformants was obtained. For heavy chain shuffling, the C6.5 V<sub>H</sub> CDR3 and light chain were cloned into a vector containing a human V<sub>H</sub> gene repertoire to create a phage antibody library of  $1 \times 10^6$  transformants. After selection, a single higher affinity light chain shuffled scFv (C6L1) was identified. C6L1 had a K<sub>d</sub> 6 times lower than C6.5. After selection, 2 higher affinity heavy chain shuffled scFv were identified. C6H2 had a K<sub>d</sub> 5 times lower than C6.5. In an attempt to further increase affinity, shuffled rearranged V<sub>H</sub> and V<sub>L</sub> genes from higher affinity scFv were combined into the same scFv. Combining the rearranged V<sub>L</sub> gene from C6L1 with the rearranged V<sub>H</sub> gene from C6H1 resulted in an scFv (C6H1L1) with lower affinity than either C6L1 or C6H1. Similarly, combining the rearranged V<sub>L</sub> gene from C6L1 with the rearranged V<sub>H</sub> gene from C6H2 resulted in an scFv (C6H2L1) with lower affinity than C6L1 or C6H2. Thus in both instances combining the independently isolated higher affinity rearranged V<sub>H</sub> and V<sub>L</sub> genes did not have an additive effect on affinity.

**Significance:** Chain shuffling was an effective technique for increasing the affinity of the c-erbB-2 (protein antigen) binding scFv C6.5. The 6 fold increase in affinity compares favorably to the 6 fold increase observed with parsimonious mutagenesis. However, we failed to see an additive effect on affinity when the V<sub>H</sub> and V<sub>L</sub> shuffled chains were combined. This is unexpected, typically the effect of mutations are additive (28, 54). The reason for the lack of additivity is unclear, but suggests that a sequential approach to chain shuffling (24) may be more prudent. More importantly, the relatively random distribution of mutations in higher affinity clones provided little useful information as to where to direct additional mutations to further increase affinity. Thus site directed mutagenesis was explored, both as technique to identify residues which modulate affinity and as a technique to increase affinity more efficiently.

### 2.3.7 Increasing the affinity of C6.5 by site directed mutagenesis (Schier et al., 1996, appendix 4 and 5).

As an alternative to random mutagenesis, mutations can be targeted (site directed mutagenesis) to amino acid residues located in the antibody combining site. Since it is difficult to make libraries greater than  $10^7$  to  $10^8$  clones, decisions must be made as to which residues to mutate, and to what extent. One approach is suggested by structural and functional analysis of the antibody combining site. Typically, 15-22 amino acids in the combining site of an antibody contact a similar number of amino acids in antigen (55). However free energy calculations and mutational analysis indicate that only a small subset of the contact residues contribute the majority of the binding energy (28, 56, 57). For the rest of the residues, a decrease in entropy accounts for most of the enthalpy decrease, resulting in no net effect on affinity (56, 57). In many instances, 'repulsive contacts' are also made, which can cost up to several kcal (56). Thus antibody affinity could be increased by exchanging low affinity or repulsive contacts for higher affinity contacts while retaining the few residues which contribute the majority of the binding energy. The problem is how to identify these residues, in the absence of high resolution structural and functional data.

Analysis of antibody combining sites indicates that the majority of the contact residues are in located in six hypervariable loops, three (L1, L2, and L3) in the light chain variable

domain ( $V_L$ ), and three (H1, H2, and H3) in the heavy chain variable region ( $V_H$ ) (reviewed in ref. (58)). The limits of the loops are defined structurally as lying outside of the  $\beta$ -sheet (59, 60) and these limits are slightly different than the complementarity determining regions (CDRs) defined by Kabat on the basis of sequence hypervariability (61). The length of human L1, L2, L3, H1, and H2 can vary from 3 to 10 amino acids, with H3 lengths as long as 18 residues (59-61). Thus up to 51 residues need to be scanned. Conventional oligonucleotide directed mutagenesis uses the nucleotides NNS to randomize each residue. All parental contacts are discarded and the number of residues that can be scanned is limited to 5, given typical transformation efficiencies. A greater number of residues can be scanned by parsimonious mutagenesis (PM), using oligonucleotides designed to minimize coding sequence redundancy and limit the number of residues which do not retain parental structural features (62). Redundancy is reduced using (doping) codons where degeneracy is equal to or only slightly larger than the subsets of amino acids encoded. Non-viable structures are minimized by using biased (spiked) nucleotide mixtures which bias for the parental amino acid and take advantage of the tendency of the genetic code to favor chemically or sterically conservative amino acid changes. In section 2.3.6A, we report how PM was used to simultaneously scan 19 residues located in loops within the CDRs of C6.5 resulting in the identification of functional residues that modulate affinity and conserved residues essential for structural integrity of the scFv. In section 2.3.6B, we report how sequential mutation of  $V_L$  and  $V_H$  CDR3 of C6.5 led to an 1100 fold increase in affinity.

### **2.3.7A. Identification of structural and functional residues in the complementarity determining regions of $V_H$ and $V_L$ domains of scFv (Schier et al., 1996, appendix 4)**

To determine the utility of PM as a scanning technique, three loops of C6.5 were simultaneously mutated by PM and the resulting gene repertoire cloned for display on the surface of phage. C6.5 mutants with 6 fold higher affinity for c-erbB-2 ( $K_d=2.4 \times 10^{-9}$  M) were selected from the library and residues within the loops important for modulation of affinity identified. This work is summarized below and described in greater detail in Schier et al, appendix 4.

The  $V_L$  domain of C6.5 is a member of the  $V_{\lambda 1}$  family, and could be modeled using the three dimensional structure of the  $V_{\lambda 1}$  domain of KOL (63). L1 consists of 9 residues, L2 of 3 residues, and L3 of 8 residues (59). The  $V_H$  domain of C6.5 is derived from the DP73 germline gene of the  $V_H5$  family (64) and could be modeled using the three dimensional structure of the  $V_H$  domain of NC41 (65). H1 consists of 7 residues, H2 of 6 residues, and H3 of 17 residues (60). Thus the loops consist of a total of 50 amino acids, too large a sequence space to search simultaneously, even using PM. L2 was excluded from PM since it is the loop that least frequently contains residues which contact antigen (58). H1 was excluded because 3 of the 7 residues (G26, F27, and F29) have structural roles and the residues at these positions are generally conserved in  $V_H$  domains (59, 60). H3 was excluded from PM due to its length. The remaining 3 loops (L1, L3, and H2) were selected for randomization by PM. All 8 residues of L3 were subjected to PM as were all 6 residues of H2. Five C-terminal residues of L1 (28-32, Kabat numbering, (61)) were subjected to PM. Residues 26 to 27b were excluded from PM since they are relatively conserved in antibody structures and are more constrained by framework contacts.

Nineteen amino acids were subjected to PM. The library was designed so that the most abundant sequences contained 5 non-parental amino acids. Thus the frequency of a non-parental amino acid at each site is 0.26 (5/19), with approximately 80% of the library containing between 2 and 7 non-parental amino acids. At each position, alternative amino acid sets ranged from 10 to 19 amino acids encoded by 12 to 32 codons. After transformation of *E. coli* TG1 (66), a library of  $1.0 \times 10^6$  clones was obtained.

The PM phage antibody library was subjected to four rounds of selection in solution on biotinylated c-erbB-2, starting with an antigen concentration of  $4.0 \times 10^{-8}$  M and decreasing to

$1.0 \times 10^{-11}$  M. This selection approach uses limiting antigen concentrations in the latter rounds to drive affinity based selection, while the high antigen concentration in early rounds ensures the capture of rare binders (25). Prior to selection, only 3/92 scFv bound c-erbB-2 by ELISA, while after 3 and 4 rounds of selection, virtually all scFv bound c-erbB-2. The dissociation rate constant ( $k_{\text{off}}$ ) was determined on native scFv in bacterial periplasm for 20 ELISA positive clones from the third and fourth rounds of selection using surface plasmon resonance in a BIAcore. After three rounds of selection, 3 of 20 scFv (12%) had a  $k_{\text{off}}$  slower than the parental scFv, while after four rounds of selection, 10/20 scFv (50%) had a slower  $k_{\text{off}}$ . All 13 scFv with a slower  $k_{\text{off}}$  were sequenced, subcloned into pUC119Hismyc (25) and purified by immobilized metal chelate chromatography, followed by gel filtration to remove any scFv aggregates. Affinities were determined for each scFv by surface plasmon resonance in a BIAcore. Two of the three scFv isolated after the third round of selection were not higher affinity than the parental scFv, while the third had an affinity 3 fold higher than parental (Table V, appendix 3). All ten scFv from the fourth round of selection had higher affinity than the parental scFv, with the best clone (C6PM6) having a 6 fold increase in affinity ( $2.4 \times 10^{-9}$  M). The results confirm the effectiveness of the selection approach to enrich for higher affinity scFv and BIAcore screening to identify higher affinity scFv. Only 2 of 13 scFv purified did not have an improved affinity. Both of these scFv were from the third round of selection. The affinity of C6PM6 ( $2.4 \times 10^{-9}$  M) compares favorably to the affinity of murine antibodies produced against the same antigen using conventional hybridoma technology (11, 67).

Sequence analysis of higher affinity scFv indicated that substitutions occurred at 10/19 (53%) of the positions, with 21/28 substitutions occurring at only 4 positions, 2 in H2, and 1 each in L1 and L3 (Table VI, appendix 3). Thus PM identified a subset of 'functional' residues whose mutation results in increased affinity. All but 1 of these 10 residues ( $V_{\lambda}$  L95) appear to have solvent accessible side chains in our C6.5 model. In contrast, two residues ( $V_{\lambda}$  N30 and  $V_{\text{H}}$  Y52) with solvent exposed side chains are 100% conserved, suggesting these are 'functional' residues which are critical for recognition.

The majority (7/9) of the conserved residues, however, appear to have a structural role in the variable domain, either in maintaining the main chain conformation of the loop, or in packing on the  $V_{\text{H}}$  domain. In the  $V_{\lambda}$  domain, residues I28, G29, W91, and D92 are present in both C6.5 and KOL (63), consistent with a structural role. The side chain of I28 is buried deep in the core of the  $V_{\lambda}$  domain between hydrophobic residues 25, 33, and 71, and is a major determinant of the main chain conformation of L1 (59). In the model of C6.5,  $V_{\lambda}$  G29,  $V_{\lambda}$  G95b, and  $V_{\text{H}}$  G53 are in turns and  $V_{\lambda}$  W91 and  $V_{\lambda}$  W96 pack against the  $V_{\text{H}}$  domain at the  $V_{\text{H}}$ - $V_{\text{L}}$  interface. Hydrogen bonds between  $V_{\lambda}$  D92 and  $V_{\lambda}$  S27a and  $V_{\lambda}$  N27b bridge L3 and L1 to stabilize the L3 and L1 conformations. The results suggest that even conservative substitution of residues known to have a structural role does not produce higher affinity antibodies. Thus, efficient *in vitro* evolution of proteins could be achieved by reducing the sequence space that requires scanning by homology modeling or sequence alignments of members of structurally related families.

#### Significance:

Homology modeling of antibody Fv fragments can be used to identify structural amino acid residues; mutation of these residues is unlikely to result in higher affinity scFv. Parsimonious mutagenesis can be used to screen a large sequence space to identify residues which modulate affinity. These residues could be selected for more thorough scanning, using a higher mutagenic rate to produce yet higher affinity antibodies.

#### **Year 2**

#### **2.3.7B Results of experiments to increase the affinity of C6.5 by site directed mutagenesis of $V_{\text{L}}$ CDR3 and $V_{\text{H}}$ CDR3.**

In parallel to the PM experiments described above, we sequentially mutated only the V<sub>L</sub> and V<sub>H</sub> CDR3 of C6.5 but at a much higher mutation frequency than used for PM. Thus many fewer residues (4-9) were mutated in a single library, but much greater sequence diversity was sampled at each position.

### 2.3.7B1 Site directed mutagenesis of V<sub>L</sub>CDR3.

Initial efforts focused on mutating V<sub>L</sub>CDR3, given its smaller size compared to the V<sub>H</sub>CDR3 (9 residues vs. 20). Since the results of the PM experiments were not known when these experiments were undertaken, modeling was not performed to identify structural residues and thus all residues in the V<sub>L</sub>CDR3 were randomized. For construction of a library of C6.5 V<sub>L</sub> CDR3 mutants, an oligonucleotide was designed (Table 1, appendix 5) which partially randomized nine amino acid residues located in V<sub>L</sub> CDR3 (residues 89-95b, Table 2, appendix 5). For the nine amino acids randomized, the ratio of nucleotides was chosen so that the frequency of wild type amino acid was 49%. After transformation, a library of  $1.0 \times 10^7$  clones was obtained. The mutant phage antibody library was designated C6VLCDR3. The C6VLCDR3 library was subjected to four rounds of selection using decreasing concentrations of biotinylated c-erbB-2 ECD, as described in ref. (68). A relatively high concentration ( $4.0 \times 10^{-8}$  M) was used for the first round to capture rare or poorly expressed phage antibodies. The concentration was decreased 40 fold for the second round ( $1.0 \times 10^{-9}$  M), and decreased a further tenfold each of the subsequent two rounds ( $1.0 \times 10^{-10}$  M, 3rd round;  $1.0 \times 10^{-11}$  M, 4th round). After each round of selection, the concentration of binding phage in the polyclonal phage preparation was determined by measuring the rate of binding of polyclonal phage to c-erbB-2 under mass transport limited conditions using SPR in a BIAcore, as described above. The results were used to guide the antigen concentration used for the subsequent round of selection. After both the third and fourth rounds of selection, 92/92 clones bound c-erbB-2 by ELISA. After the third round of selection, seven unique scFv were identified, all with higher affinity than wild type scFv. scFv had on average 1.8 amino acid substitutions/scFv, with a single substitution at residue 92 the most frequently observed mutation. These single amino acid substitutions would have occurred with a frequency of 1/12,000 in the original library, assuming equal nucleotide coupling efficiency. The average scFv affinity was  $3.6 \times 10^{-9}$  M (4.4 fold increase), with the highest affinity  $2.6 \times 10^{-9}$  M (sixfold increase). After four rounds of selection, six scFv were identified, and none of these sequences were observed in the scFv sequenced from the third round. scFv from the fourth round had on average 2.9 amino acid substitutions/scFv, with expected frequencies of between 1/590,000 and 1/24,000,000 in the original library. The average scFv affinity after the fourth round was  $1.9 \times 10^{-9}$  M (8.4 fold increase), with the highest affinity  $1.0 \times 10^{-9}$  M (16 fold increase) Table 2, appendix 5). The results demonstrate the efficiency of the selection technique for isolating very rare high affinity clones from a library. Significant sequence variability (five different amino acids) was observed at residues 93, and 94, with less variability (two different amino acids) at residues 95 and 95a. Thus, similar to the PM experiments, a subset of the randomized residues appear to be more important in modulating affinity. All but one of these four residues (V<sub>L</sub> L95) appear to have solvent accessible side chains in our C6.5 model (figure 1, appendix 5). Three of the residues randomized (A89, W91, and G96) were 100% conserved in all mutants sequenced. Two additional residues (A90S and D92E) showed only a single conservative substitution. These conserved residues appear to have a structural role in the variable domain, either in maintaining the main chain conformation of the loop, or in packing on the V<sub>H</sub> domain. Residues A89, W91, and D92 are identical in both C6.5 and KOL (Marquart *et al.*, 1980), with conservative substitutions A90S and G96A observed at the other two positions in KOL, consistent with a structural role. In the model of C6.5 G95b is in a turn and A89, A90, and W91 pack against the V<sub>H</sub> domain at the V<sub>H</sub>-V<sub>L</sub> interface (figure 1, appendix 5). Hydrogen bonds

between  $V_{\lambda}D92$  and  $V_{\lambda}S27a$  and  $V_{\lambda}N27b$  bridge L3 and L1 to stabilize the L3 and L1 conformations.

### 2.3.7B2 Site directed mutagenesis of $V_H$ CDR3.

To further increase the affinity of C6.5, we chose to mutate the  $V_H$  CDR3 of the highest affinity scFv (C6ML3-9,  $K_d = 1.0 \times 10^{-9}$  M) isolated from the C6VLCDR3 library, rather than mutate C6.5  $V_H$  CDR3 independently and combine mutants. This sequential approach was taken since the kinetic effects of independently isolated antibody fragment mutations are frequently not additive (68, 69). Due to the length of the C6.5  $V_H$  CDR3 (20 amino acids), a high resolution functional scan was performed on C6.5 scFv in an attempt to reduce the number of amino acids subjected to mutation. Residues 95-99, 100a-100d, and 100g-102 were separately mutated to alanine, and the  $K_d$  of the mutated scFv determined. Residue 100f (alanine) was not studied. Residues 100 and 100e are a pair of cysteines separated by four amino acids. A homologous sequence in the Fv KOL (63) results in a disulfide bond between the two cysteines and a four residue miniloop. Therefore the two cysteines were simultaneously mutated to serine. Results of the alanine scan are shown in Table 3, appendix 5. No detectable binding to c-erbB-2 ECD could be measured by BIAcore for C6.5H95A, C6.5W100hA, and C6.5E100jA. Three additional alanine mutants (G98A, Y99A, and F100A) yielded scFv with 20 fold to 100 fold higher  $K_d$  than wild type scFv. Substitution of the two cysteines by alanine (100, 100e) yielded an scFv with an 17.5 fold higher  $K_d$ , and a much faster  $k_{off}$  ( $1.38 \times 10^{-1} s^{-1}$ ) than wild type C6.5. The remainder of the alanine substitutions yielded only minor (0.5 to 3.7 fold) increases or decreases in  $K_d$ .

Based on the results of the alanine scan and a model of C6.5 based on the Fv KOL (Marquardt et al., 1980), residues H95A, C100, and C100e were not mutated due to their probability of having an important structural role. H95 is likely to be buried at the  $V_H$ - $V_L$  interface where it makes critical packing contacts with the  $V_L$  domain. The two cysteine residues also are likely to have a structural role in maintaining the miniloop conformation. W100a was also not mutated given the unique features of tryptophan in antibody combining sites (70). The remaining 16 amino acids were completely randomized four residues at a time in four separate C6VHCDR3 libraries (96-99, library A; 100a-100d, library B; 100f, 100g, 100i, and 100j, library C, and 100k-102, library D; see Table 4, appendix 5). After transformation, libraries were obtained with sizes  $1.7 \times 10^7$  (library A),  $1.3 \times 10^7$  (library B),  $3.0 \times 10^6$  (library C), and  $2.4 \times 10^7$  (library D). The mutant phage antibody libraries were designated C6VHCDR3 libraries A, B, C, and D. Prior to selection, the percent of clones expressing scFv which bound c-erbB-2 by ELISA was 1% for C6VHCDR3 library A, 57%, library B, 2% library C, and 3% library D. The C6VHCDR3 libraries A, B, C, and D were selected on biotinylated c-erbB-2 ECD as described above and in ref. (68), but using lower antigen concentration. The rate of binding of polyclonal phage was measured in a BIAcore to determine the antigen concentration used for the subsequent round of selection.

After four rounds of selection, positive clones were identified by ELISA and at least 24 scFv from the fourth round of selection were ranked by  $k_{off}$  using SPR in a BIAcore. The ten scFv with the lowest  $k_{off}$  from C6VHCDR3 libraries A, C, and D were sequenced. Due to the diversity of isolated scFv in C6VHCDR3 library B, 48 scFv were ranked by  $k_{off}$  using SPR, and 22 clones with the lowest  $k_{off}$  were sequenced. scFv were purified by IMAC, followed by gel filtration to remove any dimeric or aggregated scFv. The  $k_{on}$ , and  $k_{off}$  were determined by BIAcore and the  $K_d$  calculated. Very different results were obtained from the four libraries with respect to the number of higher affinity scFv isolated, and the value of the highest affinity scFv. The best results were obtained from library B (Table 4, appendix 5). Fifteen scFv were isolated with a  $K_d$  lower than wt C6ML3-9 and no wt sequences were observed. The best scFv (C6MH3-B47) had a  $K_d = 1.1 \times 10^{-10}$  M, ninefold lower than C6ML3-9 and 145 fold lower than C6.5. The

$k_{\text{off}}$  of this scFv was  $7.5 \times 10^{-5} \text{ s}^{-1}$ , tenfold lower than C6ML3-9 and 84 fold lower than C6.5. While a wide range of sequences was observed, a subset of scFv had the consensus sequence TDRT (first eight scFv, Table 4, library B, appendix 5). The consensus sequence is identical with the sequence of C6MH3-B1, which is the scFv with the lowest  $k_{\text{off}}$  ( $6.0 \times 10^{-5} \text{ s}^{-1}$ ). Five scFv were isolated that had a  $k_{\text{off}}$  2.5 to 3.75 fold lower than C6ML3-9, however expression levels were too low to obtain adequate purified scFv for measurement of the  $K_{\text{d}}$  (last five sequences, Table 4, library B, appendix 5). The next best results were obtained from library D (Table 4, library D, appendix 5). Five higher affinity scFv were isolated, with the best having a  $K_{\text{d}}$  sevenfold higher than wild type C6ML3-9. An additional scFv was isolated that had a  $k_{\text{off}}$  lower than wt scFv, however the expression level was too low to obtain adequate purified scFv for measurement of the  $K_{\text{d}}$  (last sequence, Table 4, library D, appendix 5). There was selection for a consensus mutation of Y100kW and replacement of F100I with hydrophobic methionine or leucine.

No higher affinity scFv were isolated from either the A or C libraries. From library A, 8/10 scFv were wild type, with one higher affinity scFv, a contaminant from library B. A single mutant scFv with the conservative replacement of Y99F had an apparent  $k_{\text{off}}$  2.5 times lower than wt, but expression levels were too low to obtain adequate purified scFv to measure the  $K_{\text{d}}$ . From library C, 8/10 scFv were wild type scFv, with one higher affinity scFv having mutations located in the  $V_{\text{H}}$  and  $V_{\text{L}}$  genes, but not in the region intentionally mutated. The isolated mutant scFv K100gV had a  $K_{\text{d}}$  2.7 fold lower than wt ( $k_{\text{off}}$  3.8 fold lower than C6ML3-9).

### 2.3.7B3 Effects on affinity of combining $V_{\text{H}}$ CDR3 mutations.

To further increase affinity, the sequences of the two highest affinity scFv obtained from the  $V_{\text{H}}$  CDR3B library (C6MH3-B1 or C6MH3-B47) were combined with the sequences of scFv isolated from the C6VHCDR3D library (C6MH3-D1, -D2, -D3, -D5, or -D6). An increase in affinity from wild type was obtained for all these combinations, yielding an scFv (C6-B1D3) that had a 1230 fold lower  $K_{\text{d}}$  than wild type C6.5 (Table 5, appendix 5). The extent of additivity varied considerably, however, and could not be predicted from the parental  $k_{\text{on}}$ ,  $k_{\text{off}}$ , or  $K_{\text{d}}$ . In some combinations, cooperativity was observed, with a negative  $\Delta\Delta G_{\text{I}}$ . Additional combinations were made between a previously described light chain shuffled C6.5 mutant (C6L1, sixfold decreased  $K_{\text{d}}$ , (68)) and one of two  $V_{\text{L}}$  CDR3 mutants (C6ML3-9 and C6ML3-12). These combinations yielded scFv with 49 and 84 fold improved affinity (Table 5). Introducing the same rearranged  $V_{\text{L}}$  gene into the highest affinity  $V_{\text{H}}$  CDR3 mutants (C6MH3-B1 or C6MH3-B47) resulted in decreased affinity compared to C6MH3-B1 (Table 5, appendix 5). A similar effect was described in previous work (68) when rearranged  $V_{\text{L}}$  and  $V_{\text{H}}$  genes from high affinity chain shuffled scFv obtained from parallel selection were combined.

#### Significance:

Ultra-high affinity scFv were engineered by diversifying the CDRs that comprise the center of the antibody combining site. Sequential diversification of  $V_{\text{L}}$  and  $V_{\text{H}}$  CDR3 yielded scFv with up to a 145 fold increase in affinity ( $K_{\text{d}} = 1.1 \times 10^{-10} \text{ M}$ ). Combination of these mutations with independently selected mutations located elsewhere in  $V_{\text{H}}$  CDR3 yielded an additional ninefold increase in affinity ( $K_{\text{d}} = 1.3 \times 10^{-11} \text{ M}$ ). The scFv were produced without any immunization and have higher affinity than any antibody fragments engineered *in vitro*. The results illustrate the power of diversity libraries and phage display to produce antibody fragments with affinities rarely achieved by immunization (7) and have important implications for the design of mutant phage antibody libraries (see section 2.3.7, below). Moreover, the availability of such high affinity antibody fragments may have important consequences for antibody based tumor targeting (see section 2.4, below).

### 2.3.8 Conclusions on optimal approach to increasing antibody fragment affinity

When designing a mutant phage antibody library, decisions must be made as to how and where to introduce mutations. Mutations can be randomly introduced, using either chain shuffling (23, 24), error prone PCR (27), or mutator strains (51), thus apparently mimicking the process of somatic hypermutation. These approaches have yielded large increases in affinity for hapten antigens (>100 fold) (24, 51), but results with protein binding antibody fragments have been more modest (<10 fold) ref. (27); and the work described in section 2.3.6 and Schier et al., 1996, appendix 5). Moreover, the relatively random distribution of mutations in higher affinity clones provides little useful information as to where to direct additional mutations. Alternatively, knowledge of the general structure of the Fv fragment and its complexes with antigen can be used to direct mutagenesis to the CDRs that form the contact interface between antibody and antigen.

Targeting mutations to the CDRs has previously been shown to be an effective technique for increasing antibody affinity. Yang et al. (69) increased the affinity of an anti-HIV gp120 Fab 420 fold ( $K_d = 1.5 \times 10^{-11}$  M) by mutating four CDRs in five libraries and combining independently selected mutations. We achieved three times that increase in affinity by mutating a much smaller portion of the antibody combining site contained within only 2 CDRs. Our results may be partly due to the stringent selection conditions used and the techniques used to monitor selections and screen for higher affinity scFv without the need for purification. However, the results also suggest that focusing mutations in  $V_H$  and  $V_L$  CDR3 may be a more efficient means to increase affinity.

Directing mutations into  $V_H$  and  $V_L$  CDR3 to increase affinity may initially seem at odds with studies on antibody structure and function. Although 15-22 amino acids located in loops within the CDRs typically contact antigen (55), free energy calculations and mutational analysis indicate that only a small subset of the contact residues contribute the majority of the binding energy (28, 56, 57). The high energy contact residues are more frequently located in the center of the antibody combining sites in the  $V_H$  and  $V_L$  CDR3s. Thus, mutation of  $V_H$  and  $V_L$  CDR3 is more likely to destroy high affinity contacts than mutation of other CDRs. However, these residues will be recreated, albeit at low frequency, given an adequate library size for the number of residues randomized. Mutant residues could increase affinity by introducing new contact residues (71) or by replacing low affinity (56, 57) or 'repulsive' contact residues (56) with contact residues with more favorable energetics. It appears, however, that many mutations introduced either by somatic hypermutation *in vivo* (72) or mutagenesis *in vitro* (28) exert their effect on affinity indirectly, in many instances by precisely positioning the side chains of contact residues for optimal electrostatic, hydrogen bonding, and van der Waals interactions (70). Mutation of non-contact CDR residues located close to high energy contact CDR residues may be more likely to exert this indirect effect. The importance of the CDR3s as sites for mutagenesis is also supported by the work of Yang et al. (69) who created separate libraries of  $V_L$  CDR1,  $V_L$  CDR3,  $V_H$  CDR1, and  $V_H$  CDR3 mutants. The largest increases in affinity were 7.9 and 7.7 fold from sequential mutation of two separate regions of  $V_H$  CDR3, resulting in a 63 fold increase in affinity over wild type Fab. Mutation of  $V_L$  CDR3 resulted in the next largest increase in affinity over wild type (5.6 fold).

Directing mutations into  $V_H$  and  $V_L$  CDR3 to increase affinity may also appear to be different than the locations where mutations are directed and accumulate during somatic hypermutation *in vivo*. Germline diversity is greatest in the center of the antibody combining site (73), particularly  $V_H$  and  $V_L$  CDR3, where tremendous sequence diversity is generated by recombination, N segment addition, and joining diversity. Somatic hypermutation extends sequence diversity to CDR residues located more peripherally in the antibody combining site (73). On closer inspection, however, striking similarities exist between our results and somatic hypermutation *in vivo*. Nucleotide substitutions *in vivo* are not targeted randomly, but rather occur at specific sequence hotspots intrinsic to the mutational process, for example at serines encoded by the nucleotides AGY but not at serines encoded by TCN (74, 75). Serines encoded by AGY have previously been shown to predominate over serines encoded by TCN in CDR1 and 2

of the V<sub>H</sub> domain and CDR1 of the V<sub>K</sub> domain, but not in the frameworks (75, 76). The germline gene segments encoding the V<sub>K</sub> and V<sub>λ</sub> CDR3s are similarly biased to contain a high proportion of AGY serines (figure 5, appendix 5). The CDR3 AGY/TCN ratio is 9.7 for the V<sub>K</sub> gene segments and 5.4 for the V<sub>λ</sub> gene segments. These values are comparable to the AGY/TCN ratios observed for V<sub>H</sub> CDR1 (20.3), V<sub>H</sub> CDR2 (2.28), and V<sub>K</sub> CDR1 (8.75) and are greater than values observed for framework residues (0.3 to 0.67) (76). Thus the sequences encoding the V<sub>L</sub> CDR3 have evolved to be targets of the somatic hypermutation machinery. Accordingly, an extensive analysis of the location of mutations introduced by somatic hypermutation into the germline V<sub>K</sub> genes identified CDR3 as a frequent site of mutagenesis (73). Within V<sub>K</sub> CDR3, residues 89 to 91 are most conserved, with the highest frequency of mutation observed at residue 93 (Tomlinson *et al.*, 1996). Similarly, during *in vitro* affinity maturation of the C6.5 V<sub>λ</sub> CDR3, we observed conservation of residues 89 to 92, and 95b (this work) and 96 (ref. (77)). In contrast, substitution occurred at residues 93-95a, with the highest frequency of mutation at residue 93. The location of mutations parallels exactly the distribution of AGY serines within the germline V<sub>λ</sub> CDR3 genes (figure 5, appendix 5).

Such a detailed analysis is more difficult for V<sub>H</sub> CDR3 since germline D gene segment assignment is frequently not possible, and the V<sub>H</sub> CDR3 loop cannot be accurately modeled. In 33 published D gene segments (61), the ratio of AGY/TCN serines is 2.5/1, a value closer to that observed for CDRs (2.3 to 20.3) than for framework residues (0.3 to 0.67). Thus portions of the V<sub>H</sub> CDR3 also appear to be targets for somatic hypermutation *in vivo*. In the case of the C6.5, the sequence motif C<sub>2</sub>SSNC located within the V<sub>H</sub> CDR3 is encoded by the germline D gene segment D1 (61) which encodes the sequence CSSTSC. In the germline gene, all 3 serines are encoded by AGY and in C6.5 these 3 residues were hotspots for substitutions which increased affinity. Moreover, an extremely wide range of amino acid residues were tolerated between the 2 cysteines, as evidenced by the alanine scan results and the fact that 57% of unselected scFv bound antigen. In contrast, only 1-2% of unselected scFv from the 3 other V<sub>H</sub> CDR3 libraries bound antigen. One possible explanation is that the disulfide bond formed by the cysteines stabilizes this region of the V<sub>H</sub> CDR3. Regardless, five of the 33 D gene segments encode a CXXXXC or CXXXC (Kabat *et al.*, 1991) in the preferred reading frame (78, 79) suggesting that the motif has useful properties as a component of the primary immune repertoire. If so, then use of similar motifs could prove useful in construction of semi-synthetic antibody libraries (80).

Modeling the location of the observed mutations in V<sub>L</sub> CDR3 on the Fab structure KOL, where the V<sub>λ</sub> is derived from the same germline gene as C6.5, suggested a basis for the pattern of conservation and substitution we observed. The conserved residues appear to have a significant structural role in the variable domain, either in maintaining the main chain conformation of the loop, or in packing on the V<sub>H</sub> domain. In our model, the side chain of W91 is buried at the V<sub>H</sub>-V<sub>L</sub> interface and by the long V<sub>H</sub> CDR3 of KOL. In other structures, the side chain of V<sub>L</sub>91 is at least partially solvent accessible, and frequently contacts antigen (70, 73). In contrast to the extensive packing of the side chains of conserved residues, all but one of the four most extensively substituted residues appear to have solvent accessible side chains. A similar pattern was also observed during parsimonious mutagenesis of C6.5 V<sub>L</sub> CDR1 and V<sub>H</sub> CDR2 (77). Residues within the CDRs with structural roles were conserved, while substitutions were largely confined to residues with solvent accessible side chains. Analysis of the location of substitutions in V<sub>H</sub> and V<sub>L</sub> genes mutated *in vivo* also indicates that residues involved in maintaining the main chain conformation rarely undergo non-conservative substitution (64, 81).

The previous analysis suggests a mutagenesis strategy for efficiently increasing antibody fragment affinity. Mutagenesis is directed into V<sub>L</sub> and V<sub>H</sub> CDR3 sequentially, rather than by parallel evolution of the two CDRs. These two CDRs pack on each other, and mutations isolated in parallel are likely to not be additive (69). Mutagenesis is initially directed into V<sub>L</sub> CDR3 due to the limited main chain conformations (59, 81) and the ability to model the CDR on a homologous Fv or Fab structure. Modeling should be used to identify CDR residues that are

likely to have a structural role, either in maintaining the main chain conformation or in packing against the V<sub>H</sub> domain. These residues are conserved, leaving at most four to five residues which can be completely randomized in a reasonably sized library ( $10^7$  to  $10^8$  member). The highest affinity V<sub>L</sub> CDR3 mutant is then used as a template for V<sub>H</sub> CDR3 mutagenesis. Given the length of V<sub>H</sub> CDR3s, it is likely that it will not be possible to sample the entire sequence space simultaneously. Instead residues are randomized four to five at a time, and independently selected mutations combined. In the work described here, we have observed complete conservation of the four glycine and two tryptophan residues randomized. In the CDRs, glycine residues are typically key residues in turns, and the chemical properties of tryptophan make it a frequent structural or high energy contact residue (70). Thus conservation of these two residues when randomizing V<sub>H</sub> CDR3 should be considered, if sequence space is limiting. Since combination of independently selected mutations may not be additive, it may be more prudent to simultaneously scan all residues at a low mutation frequency (parsimonious mutagenesis) to identify residues that modulate affinity, and structural and functional residues that are conserved. Residues identified as modulating affinity would then be completely randomized in a second library. Alanine scanning appears to be useful only to identify essential contact and structural residues, but not for predicting which residues would yield higher affinity when mutated (figure 2, appendix 5). If necessary, affinity could be increased further by mutating the other CDRs. Particularly suitable might be V<sub>H</sub> CDR1 and V<sub>L</sub> CDR1. These CDRs appear to be more important in modulating affinity during *in vivo* affinity maturation, based both on the higher frequency of AGY serines in the germline (73). Modeling should be performed to identify structural residues to be conserved, and residues with solvent accessible side chains, which would be mutated.

#### 2.4. Effect of scFv affinity on *in vitro* cell binding and *in vivo* biodistribution

The availability of scFv with a wide range of affinities to identical epitopes on the same antigen permit for the first time an accurate analysis of the effect of affinity on *in vivo* tumor targeting. First, we performed cell surface retention assays to verify that differences in affinity measured by SPR in the BIAcore reflected similar differences in affinity for the antigen as expressed on tumor cells. Since differences in affinity were largely due to differences in  $k_{off}$ , we measured the cell surface retention of biotinylated scFv. The retention of biotinylated C6.5 ( $K_d = 1.6 \times 10^{-8}$  M), C6ML3-9 ( $K_d = 1.0 \times 10^{-9}$  M), and C6MH3-B1 ( $K_d = 1.6 \times 10^{-10}$  M) scFv on the surface of SK-OV-3 cells expressing c-erbB-2 was determined (see Materials and Methods section of Schier et al, appendix 5 for methodology). The half life ( $t_{1/2}$ ) of the scFv on the cell surface was much less than 5 min for C6.5, 11 min for C6ML3-9, and 102 min for C6MH3-B1 (figure 2, appendix 5). These values agree closely with the  $t_{1/2}$  calculated from the  $k_{off}$  as determined by SPR in a BIAcore (1.6 min for C6.5, 13 min for C6ML3-9, and 135 min for C6MH3-B1; figure 3, appendix 5). The anti-digoxin scFv 26-10 (Huston *et al.*, 1988) was used as negative control, and no binding to c-erbB-2 ECD in a BIAcore or to c-erbB-2 on SK-OV-3 cells was observed.

To determine the impact of affinity on tumor retention, the biodistribution of  $^{125}I$ -C6.5 based scFv was determined in *scid* mice bearing subcutaneous SK-OV-3 tumors. scFv were labeled with  $^{125}I$  using the chloramine-T method as previously described (11). The quality of the radiopharmaceuticals was evaluated by HPLC, SDS-PAGE and in a live cell binding assay. The HPLC analysis was performed using a Superdex 75 column. Eluted fractions were collected and counted in a gamma well counter (11). The elution profiles consistently demonstrated that greater than 99% of the radioactivity was associated with the protein peak. The radioiodinated scFv were evaluated by SDS-PAGE with detection by autoradiography. Greater than 98% of the non-reduced  $^{125}I$ -scFv preparations migrated on SDS-PAGE as approximately 26 kDa. The immunoreactivity of the radiopharmaceuticals was determined in a live cell binding assay utilizing c-erbB-2 positive SK-OV-3 cells (11). The results of the live cell

binding assays reflected the affinity of each clone for c-erbB-2. The activity associated with the cell pellet was 61.2 %, 59.0 %, 5.3 %, and 2.7 %, respectively for C6ML3-9, C6.5, C6G98A and 26-10.

Biodistributions were determined on four to six week old inbred C.B17/Icr-*scid* mice.  $2.5 \times 10^6$  SK-OV-3 cells in log phase were implanted subcutaneously on the abdomens of the mice. After 6-8 weeks the tumors had achieved sizes of about 100 mg and Lugol's solution was placed in the drinking water to block thyroid accumulation of radioiodine. Two days later, biodistribution studies were initiated. 25  $\mu$ g (100  $\mu$ l) of  $^{125}$ I-scFv (C6G98A ( $K_d = 3.2 \times 10^{-7}$  M), C6.5 ( $K_d = 1.6 \times 10^{-8}$  M), C6ML3-9 ( $K_d = 1.0 \times 10^{-9}$  M), or 26-10 (digoxin binding scFv control)) was administered to cohorts of 6 inbred *scid* mice by tail vein injection.

The biodistribution studies revealed a close correlation between the affinity and the %ID/g of the radioiodinated scFv retained in tumor at 24 hours (figure 1, page 23). The greatest degree of tumor retention was observed with  $^{125}$ I-C6ML3-9 ( $1.42 \pm 0.23$  %ID/g). Significantly less tumor retention was achieved with  $^{125}$ I-C6.5 ( $0.80 \pm 0.07$  %ID/g) ( $p=0.03$ ). Finally, the tumor retention of the lowest affinity clone  $^{125}$ I-C6G98A ( $0.19 \pm 0.04$  %ID/g) was significantly less than that of C6.5 ( $p=0.00001$ ) and was identical to that of the negative control  $^{125}$ I-26-10. The tumor:organ ratios also reflected the greater retention of higher-affinity species in tumor. For example, tumor:blood ratios of 17.2, 13.3, 3.5 and 2.6, and tumor to liver ratios of 26.2, 19.8, 4.0 and 3.1 were observed for C6ML3-9, C6.5, C6G98A and 26-10, respectively. Similar differences were observed in the four hour %ID/gm tumor (0.69% for C6.5G98A, 2.15% for C6.5, and 3.12% for C6ML3-9).

In a separate set of experiments, the 24 hour tumor retention of the highest affinity scFv (C6MH3-B1,  $K_d = 1.6 \times 10^{-10}$  M and C6MH3-B1D3,  $K_d = 1.3 \times 10^{-11}$  M) was compared with the 24 hour retention of C6ML3-9 (figure 2, page 23). While 24 hour retention continued to increase with increasing affinity, the differences were not statistically significant.

#### Significance:

These results demonstrate that selective tumor retention of scFv molecules correlates with their affinity. While the percentage increase in retention at 24 hours is significant (more than 2 fold), the actual amount (<2% ID/gm) is not large. Calculated areas under the curve (AUC) for tumor:blood for the best scFv is only 2:1. It is estimated that a ratio of 4:1 would be required to provide predicted therapeutic dosimetry for radioimmunotherapy. It is not clear from the experiments performed to date why a greater degree of retention is not observed. One possibility is that the rapid clearance of the molecules results in an inadequate blood concentration and gradient for diffusion into the tumor. Alternatively, we are using the radiolabel as an indicator of scFv presence in the tumor. Significant degrees of dehalogenation can occur, especially with chloramine T labeling. This would result in underestimation of the tumor retention. Differences in scFv stability could also account for failure to observe greater increases in tumor targeting with the ultra high affinity scFv. These alternative explanations will be answered in subsequent experiments (see conclusions below).

#### **2.5. Creation of a bivalent C6.5 (scFv)<sub>2</sub> diabody and its' *in vitro* cell binding and *in vivo* tumor targeting properties.**

In immunodeficient mice, larger antibody molecules such as IgG and F(ab')<sub>2</sub> fragments are retained at high levels in human tumor xenografts with a low degree of specificity (82, 83), while smaller molecules such as scFv, (scFv')<sub>2</sub> and Fab are retained in tumor at comparatively lower levels with greatly improved specificity (11, 83-85). The most prominent determinant of the above targeting properties is the size of the antibody-based molecule relative to the renal threshold for first pass clearance. Another important feature of antibody-based molecules is valence, as significantly greater tumor retention has been associated with multivalent binding to

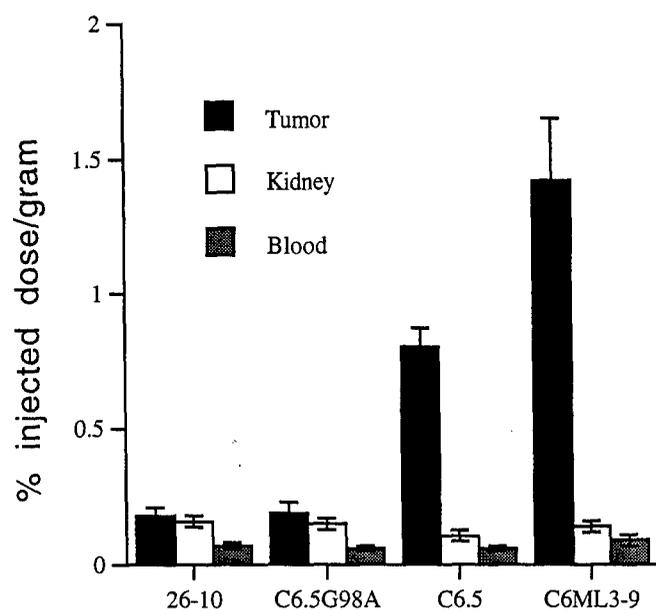


Figure 1. 24 hour tumor, kidney and blood retention of  $^{125}\text{I}$ -scFv in scid mice bearing subcutaneous c-erbB-2 expressing SK-OV-3 tumors. 26-10 = control anti-digoxin scFv; C6.5G98A = anti-c-erbB-2 scFv with  $K_d = 3.2 \times 10^{-7}$  M; C6.5 = anti-c-erbB-2 scFv with  $K_d = 1.6 \times 10^{-8}$  M; C6ML3-9 = anti-c-erbB-2 scFv with  $K_d = 1.0 \times 10^{-9}$  M. Specific tumor retention increases significantly with increasing affinity.

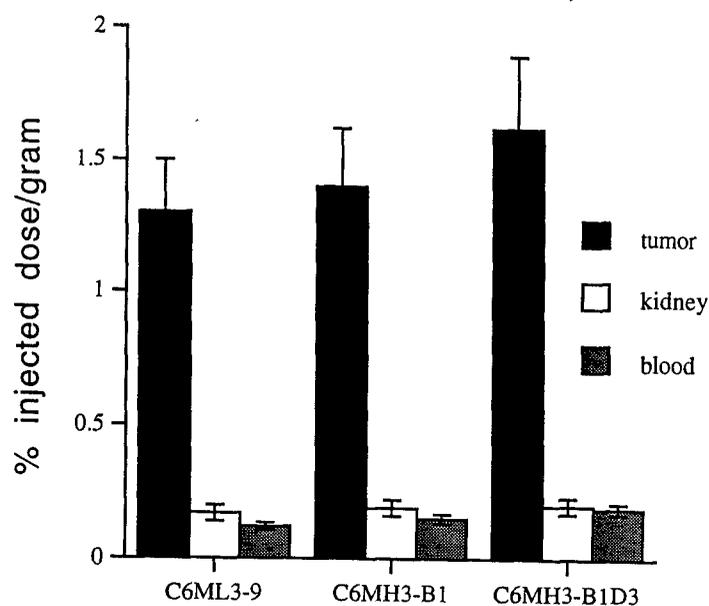


Figure 2. 24 hour tumor, kidney and blood retention of  $^{125}\text{I}$ -scFv in scid mice bearing subcutaneous c-erbB-2 expressing SK-OV-3 tumors. C6ML3-9 = anti-c-erbB-2 scFv with  $K_d = 1.0 \times 10^{-9}$  M; C6MH3-B1 = anti-c-erbB-2 scFv with  $K_d = 1.6 \times 10^{-10}$  M; C6MH3-B1D3 = anti-c-erbB-2 scFv with  $K_d = 1.3 \times 10^{-11}$  M. Specific tumor retention increases with increasing affinity, but the differences are not statistically significant.

target antigen (11, 83). To begin to systematically examine the effect of valence on antibody fragment targeting, a C6.5 (scFv)<sub>2</sub> diabody was created and its' *in vitro* binding characteristics and *in vivo* distribution examined in scid mice bearing SK-OV-3 tumors.

Diabodies are scFv dimers where each chain consists of a V<sub>H</sub> domain connected to a V<sub>L</sub> domain using a peptide linker which is too short to permit pairing between domains on the same chain (37). Instead, pairing occurs between complementary domains of another chain, creating two binding sites (figure 3, page 26). To create the C6.5 diabody, the C6.5 V<sub>H</sub> and V<sub>λ</sub> genes were joined together by PCR splicing by overlap extension using an oligonucleotide which encoded a 5 amino acid linker (G<sub>4</sub>S) between the C-terminus of the V<sub>H</sub> and the N-terminus of the V<sub>λ</sub> gene. The resulting diabody gene was cloned into pUC119mycHis (25). Native diabody was expressed and purified from the bacterial periplasm using IMAC followed by FPLC size exclusion chromatography using a Superdex 200 column as previously described for C6.5 scFv (25). The C6.5 diabody eluted as a single peak of approximately 50 kDa, with no evidence of unassociated monomer (figure 3, page 26). Typical yields of native C6.5 diabody after IMAC and HPLC purification was 1.0 mg/L.

The affinity of the C6.5 diabody for the c-erbB-2 ECD was determined using surface plasmon resonance in a BIAcore as previously described for the C6.5 scFv except that only 600 RU of c-erbB-2 ECD was coupled to the BIAcore flowcell surface. Results are shown in Table 3 and figure 4 (page 26). The K<sub>d</sub> of the C6.5 diabody for c-erbB-2 ECD was 40-fold lower than the K<sub>d</sub> of the C6.5 scFv, largely due to reduction in k<sub>off</sub>, which correlated with a retention t<sub>1/2</sub> of 43 minutes, compared to 1.8 minutes for the scFv.

**Table 3. Affinity and binding kinetics of C6.5 scFv and C6.5 diabody as determined by surface plasmon resonance in a BIAcore.** Kinetics of C6.5 scFv measured using a flowcell with 1400 RU of c-erbB-2 coupled and kinetics of C6.5 diabody measured using a flowcell with 600 RU of c-erbB-2 coupled.

Clone	K <sub>d</sub> (M)	k <sub>on</sub> (M <sup>-1</sup> s <sup>-1</sup> )	k <sub>off</sub> (s <sup>-1</sup> )
C6.5 scFv	1.6 × 10 <sup>-8</sup>	4.0 × 10 <sup>5</sup>	6.3 × 10 <sup>-3</sup>
C6.5 diabody	4.0 × 10 <sup>-10</sup>	6.7 × 10 <sup>5</sup>	2.7 × 10 <sup>-4</sup>

In the BIAcore, the c-erbB-2 ECD is chemically coupled to a three dimensional matrix of carboxymethyl dextran which could bear little resemblance to the organization of c-erbB-2 on the cell surface. Therefore, the biologic relevance of the increased affinity of the diabody was determined in an *in vitro* cell surface retention assay. Biotinylated C6.5 diabody or scFv were incubated with SK-OV-3 cells at saturating concentrations for 30 min. in the presence of sodium azide. The cells were washed and incubated at 37°C with frequent washing to remove dissociated biotinylated scFv or diabody. Aliquots were taken at various intervals, fixed with paraformaldehyde, treated with streptavidin-phycoerythrin and analyzed by flow cytometry to determine the amount of antibody fragment bound. Significantly longer retention of the C6.5 diabody was observed compared to the C6.5 scFv (t<sub>1/2</sub> scFv = 2.5 min. vs. t<sub>1/2</sub> diabody = 5 hours (p < 0.001) (figure 5, page 27). The results compare to t<sub>1/2</sub> values calculated from the k<sub>off</sub> measured in the BIAcore of 1.8 minutes for the scFv and 43 minutes for the diabody. Thus the increase in apparent affinity resulting from avidity was much greater on the cell surface than in the carboxymethyl dextran surface of the BIAcore.

The *in vivo* targeting potential of the C6.5 diabody was assessed in scid mice bearing subcutaneously. SK-OV-3 tumors overexpressing c-erbB-2. The tumor, blood, and organ retention of radioiodinated C6.5 diabody was determined at 1, 4, 24, 48 and 72 hours after intravenous administration (Table 4). Following the injections, the diabody displayed a rapid equilibration phase and subsequent slower elimination phase from circulation; this pattern is characteristic of small scFv-based molecules (figure 6, page 27). In contrast to the blood retention properties of the diabody, the quantity retained in tumor rose from 6.9 %ID/g at one

hour post injection to a peak of 10.1 %ID/g at 4 hours post injection and slowly decreased to 6.5 %ID/g and 1.4 %ID/g at 24 and 72 h respectively (figure 6, page 27). Calculations of the cumulative residence of the radioiodinated diabody, expressed as AUCs, were performed to determine predictive therapeutic potential for this molecule. Over the course of the study favorable tumor to organ AUC ratios were observed for a number of organs, including liver (3.0), spleen (6.6), bone (13.1), kidneys (2.6) and blood (3:1) (Table 4). While the activity in the bone marrow compartment is difficult to measure directly, it is routinely estimated based upon the observation that one fourth of the bone marrow compartment is composed of blood. As c-erbB-2 is not expressed on cells in the marrow, the diabody will not specifically bind to them. Therefore, the radioiodinated diabody present in the marrow compartment can be solely attributed to that present in the blood. Accordingly, the tumor to bone marrow ratio was estimated as 12:1 (25% of the tumor:blood ratio).

**Table 4. Evaluation of  $^{125}\text{I}$ -C6.5 diabody in tumor bearing scid mice.** C.B17/ICR-scid mice bearing 50-200 mg subcutaneous SK-OV-3 tumors were utilized. Cohorts of 5 mice per time point were given 20 mg of  $^{125}\text{I}$ -C6.5 diabody, and the tumor, blood, and normal tissue retention determined and expressed as a percentage of the injected dose per gram of tissue (%ID/gm) or per ml of blood (%ID/ml). Tumor:organ ratios are presented in parentheses. The cumulative diabody retention (AUC) in each tissue was determined and used to calculate the tumor to organ AUC. In a simultaneous study, the 24 hour biodistribution of C6.5 scFv was determined for 5 mice.

	C6.5 diabody						AUC	T:O AUC	C6.5 scFv
	1 hour	4 hours	24 hours	48 hours	72 hours	24 hours			
Tumor	6.9	10.1	6.5	2.4	1.4	405		1.0	
Blood	21.5 (0.3)	6.7 (1.5)	0.7 (9.5)	0.1 (22.5)	<0.1 (24)	133	3.0	0.1 (19.1)	
Liver	5.7 (1.2)	2.8 (3.6)	(0.3) (21)	0.1 (22)	0.1 (15)	137	3.0	<0.1 (25)	
Kidneys	16.9 (0.4)	5.2 (1.9)	1.1 (6.0)	0.4 (6.7)	0.3 (4.5)	153	2.6	0.2 (6.3)	
Bone	2.3 (3.3)	1.9 (6.0)	0.1 (40.6)	<0.1 (84)	<0.1 (65)	31	13.1	<0.1	
Muscle	1.2 (5.8)	1.5 (7.1)	0.2 (34.3)	<0.1 (44)	0.1 (53)	37	10.9	<0.1	

Biodistribution of the  $^{125}\text{I}$ -C6.5 scFv was performed at 24 hours after administration for comparative purposes and was found to be virtually identical to that previously reported for this and other scFv monomers of similar affinity, with 1 %ID/g retained in tumor.

#### Significance:

Bivalent C6.5 diabody was expressed and purified in high yield from *E. coli* as native protein without refolding. Compared with the scFv from which it was derived, the diabody exhibited a significantly lower  $K_d$  and slower  $k_{off}$  from c-erbB-2 either immobilized on a BIAcore sensor chip or as expressed on the surface of tumor cells. *In vivo*, radioiodinated C6.5 diabody displayed an excellent balance of quantitative tumor deposition and specificity. Peak tumor values of 10 %ID/g were observed at four hours after intravenous administration and persisted through 24 hours (6.5 %ID/g) and 72 hours (1.2 %ID/g) post injection. In contrast, the diabody was rapidly cleared from the circulation and antigen-negative organs, since its molecular weight (55 kDa) is less than the renal threshold. As a result, significantly more diabody was retained in tumor than any other organ at all but the earliest time points studied. This yielded tumor:blood AUC of 3:1. This represents a significant improvement over those observed with other antibody-based molecules including scFv, (scFv) $_2$ , Fab, F(ab') $_2$ , and IgG. The AUC ratios can be used to predict the therapeutic potential of diabodies as vehicles for radioimmunotherapy, which is dependent upon delivering lethal doses of radiation to tumor without exceeding the doses tolerated by the myelosuppressive organs such as the bone marrow (200-300 cGy) and organs involved in the catabolism of the radiopharmaceutical such as the kidneys (1500 cGy) and the liver (4000 cGy). As the delivery of tumor doses of 4000 cGy

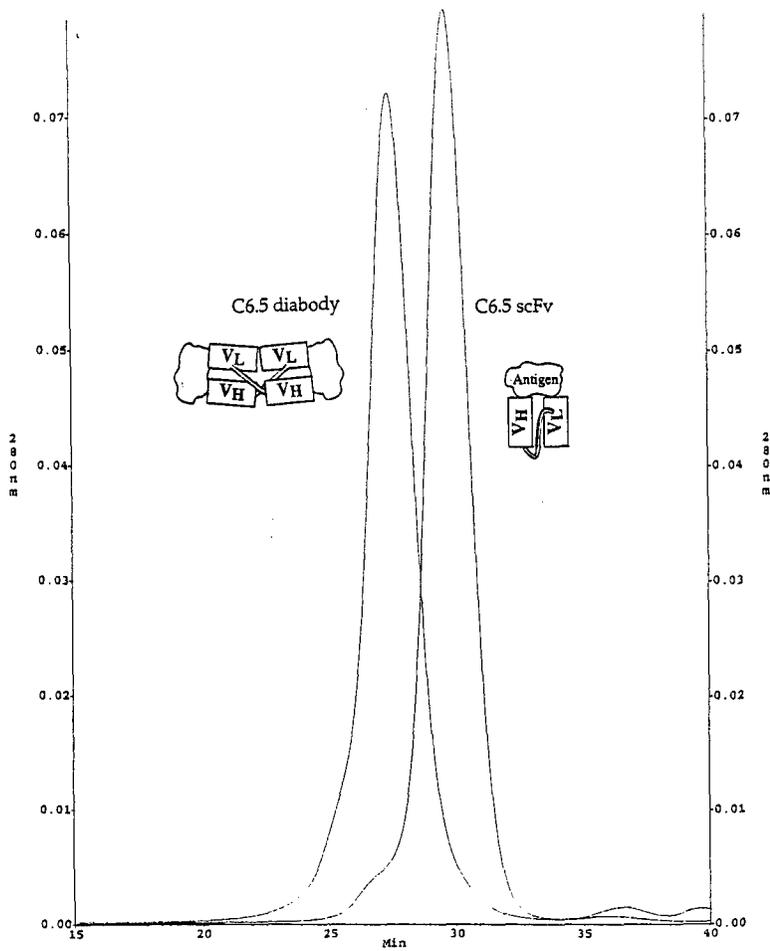


Figure 3. Gel filtration analysis of IMAC purified C6.5 scFv and C6.5 diabody. The scFv elutes as a single peak with approximate size of 25 kDa and only minimal evidence of non-covalent dimerization. The C6.5 diabody elutes as a single peak with an approximate size of 50 kDa, with no evidence of unassociated monomer or aggregation.

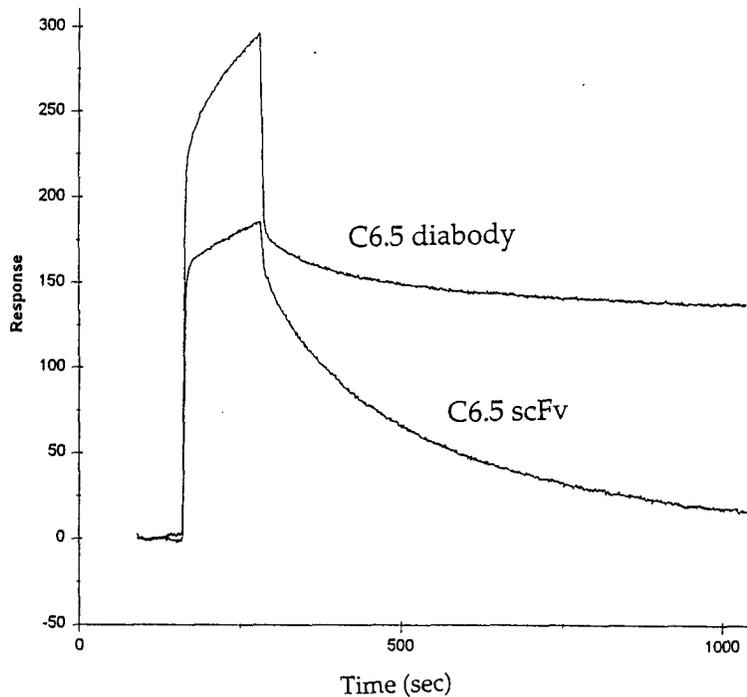


Figure 4. Sensorgrams of C6.5 scFv and diabody binding and dissociation from a c-erbB-2 coupled BIAcore flowcell surface. Monovalent C6.5 scFv dissociates rapidly from c-erbB-2 whereas the C6.5 diabody dissociation is 40 fold slower.

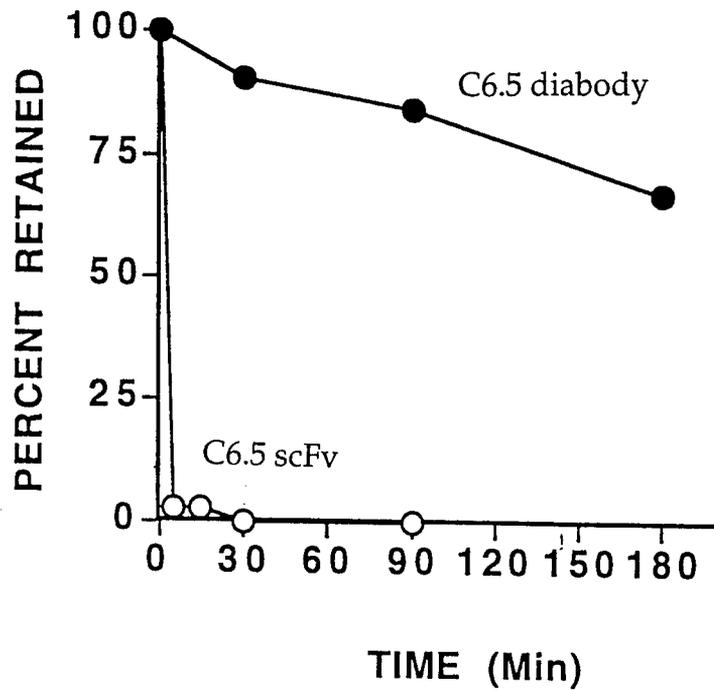


Figure 5. Retention of biotinylated C6.5 scFv and C6.5 diabody on the surface of SK-OV-3 cells. Retention is expressed as the percentage of the value at time = 0.

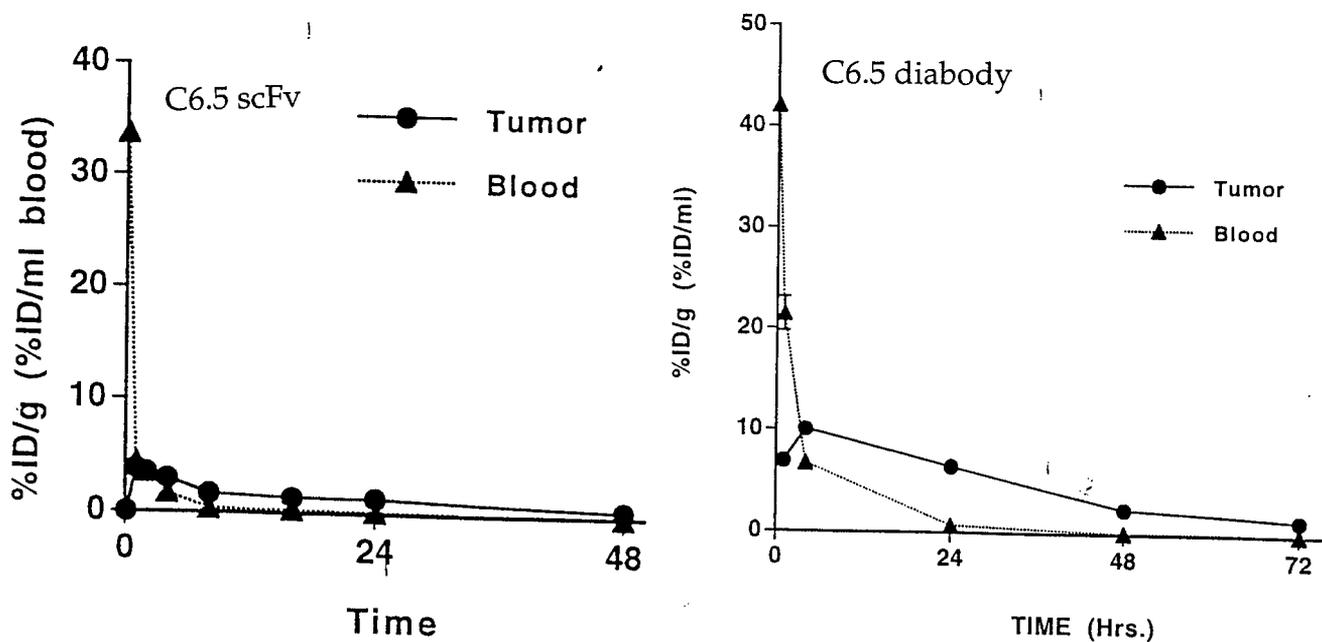


Figure 6. Tumor and blood retention of  $^{125}\text{I}$  labeled C6.5 and C6.5 diabody in scid mice bearing subcutaneous SK-OV-3 tumors.

are generally accepted as tumorogenic, tumor:organ ratios of at least 3:1 (blood), 13:1 (bone marrow), 1:1 (liver) and 2.6:1 (kidney) are likely to be necessary for therapeutic efficacy without bone marrow support. In this study with the C6.5 diabody, we calculated tumor to organ ratios ranging from 3:1 to 13:1. The tumor:bone marrow estimate of 12:1 and tumor:kidney value of 2.6:1 approximated those required for successful RIT. Thus the C6.5 diabody provides AUC ratios which are close to those required of a therapeutic molecule for radioimmunotherapy.

### 3. Conclusions

1. A large ( $7.0 \times 10^9$  member) phage antibody library has been created which can provide panels of human antibodies to purified antigens with affinities comparable to the affinities of antibodies produced by murine immunization. Current selection methodologies, however, do not permit the isolation of tumor cell specific antibodies when the libraries are selected on intact cells. To produce antibodies to unique tumor cell surface antigens, efforts in the coming years will be directed to: 1) optimization of selection techniques; and 2) creation of libraries enriched for binders to breast tumor antigens. To optimize selection techniques, we will use c-erbB-2 binding phage antibodies (see below) as a model for cell surface selections. Phage libraries will be 'spiked' with low concentrations of C6.5 based c-erbB-2 binding scFv, and a variety of depletion and enrichment schemes utilized employing c-erbB-2 positive and c-erbB-2 negative cell lines. This will permit determination of the optimal technique for cell surface selection of phage antibodies. Two approaches will be used to increase the likelihood of producing antibody fragments that bind to novel and relevant breast tumor antigens. First, phage antibody libraries enriched for antibodies binding breast tumor antigens will be created by immunizing mice with two different breast tumor cell lines (MDA MB231 and ZR-75-1). In a separate set of experiments, a subtractive immunization technique will be used to deplete the murine repertoire of antibodies binding antigens which are not tumor specific. This will be accomplished by first immunizing mice with the normal breast cell line HBL 100, followed by administration of cyclophosphamide to kill off B-cells stimulated to proliferate in response to normal cell antigens. After the drug is allowed to clear, the mice will be immunized with the tumor cell line. Spleens will be harvested and used to prepare phage antibody libraries.
2. Methodologies were developed to select rare higher affinity antibodies from phage antibody libraries. Essential parameters included: 1) use of soluble antigen in limiting concentration; 2) monitoring the stringency of selection using either surface plasmon resonance or ELISA; and 3) ensuring that the highest affinity antibodies were eluted from the affinity matrix.
3. A scanning mutagenesis technique, parsimonious mutagenesis, can be used to both increase antibody affinity, and to identify functional amino acid residues which modulate affinity and structural residues that should be conserved in subsequent libraries. Mutation of these residues at a higher frequency results in even higher affinity antibodies. In addition, the approach is considerably more efficient than traditional alanine scanning, where each residue is mutated individually.
4. Introduction of mutations into the center of the antibody combining site ( $V_H$  and  $V_L$  CDR3) appears to be an extremely effective means of increasing antibody affinity. Using this approach, we were able to increase affinity of a breast tumor targeting antibody (C6.5) more than 1200 fold to a  $K_d = 1.3 \times 10^{-11}$  M. This represents the highest affinity tumor targeting antibody produced by any technique. In addition, the range of scFv affinity mutants, which differ by at most by 10 amino acids and which bind to identical epitopes, permits delineation of the effect of antibody affinity, size, and valency on tumor targeting.
5. Increased scFv affinity correlated with greater retention of antibody on cells in vitro, and in tumors in scid mice. While the increase in tumor retention of scFv is statistically significant, the actual quantity is still quite low (<2% ID/gm) and the tumor:blood AUC is inadequate for successful radioimmunotherapy. It is not clear from the experiments performed to date

why a greater degree of retention is not observed. One possibility is that the rapid clearance of the molecules results in an inadequate blood concentration and gradient for diffusion into the tumor. Alternatively, we are using the radiolabel as an indicator of scFv presence in the tumor. Significant degrees of dehalogenation can occur, especially with chloramine T labeling. This would result in underestimation of the tumor retention. Differences in scFv stability could also account for failure to observe greater increases in tumor targeting with the ultra high affinity scFv. These alternative explanations will be answered in subsequent experiments. Stability of the radiolabel will be measured by TCA precipitation and chromatography studies of serum at various time points after injection of  $^{125}\text{I}$ -C6.5 into mice. Immunoreactivity will also be measured to determine the stability of the molecules. Rapid clearance is being addressed by construction of size and valence derivatives of the scFv.

6. Creation of divalent scFv diabodies led to large quantitative increases in antibody fragment tumor retention, with AUCs approaching those required for successful RIT. Additional diabodies will be constructed from higher affinity C6.5 scFv and in vitro cell binding and in vivo tumor targeting determined. It is expected that higher affinity diabodies will result in improved targeting and tumor:blood AUC ratios, but this hypothesis needs to be tested.

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Appendix 1

Schier RS, Marks JD, Wolf EJ, Apell G, Wong C, McCartney JE, Bookman M, Huston J, Houston LL, Weiner LM, and Adams GP. *In vitro* and *in vivo* characterization of a human anti-c-erbB-2 single-chain Fv isolated from a filamentous phage antibody library. *Immunotechnology*. 1: 73-81.



## In vitro and in vivo characterization of a human anti-c-erbB-2 single-chain Fv isolated from a filamentous phage antibody library

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### Abstract

**Background:** Antibody-based reagents have failed to live up to their anticipated role as highly specific targeting agents for cancer therapy. Targeting with human single-chain Fv (sFv) molecules may overcome some of the limitations of murine IgG, but are difficult to produce with conventional hybridoma technology. Alternatively, phage display of antibody gene repertoires can be used to produce human sFv. **Objectives:** To isolate and characterize human single chain Fvs which bind to c-erbB-2, an oncogene product overexpressed by 30-50% of breast carcinomas and other adenocarcinomas. **Study design:** A non-immune human single-chain Fv phage antibody library was selected on human c-erbB extracellular domain and sFv characterized with respect to affinity, binding kinetics, and in vivo pharmacokinetics in tumor-bearing scid mice. **Results:** A human single-chain Fv (C6.5) was isolated which binds specifically to c-erbB-2. C6.5 is entirely human in sequence, expresses at high level as native protein in *E. coli*, and is easily purified in high yield in two steps. C6.5 binds to immobilized c-erbB-2 extracellular domain with a  $K_d$  of  $1.6 \times 10^{-8}$  M and to c-erbB-2 on SK-OV-3 cells with a  $K_d$  of  $2.0 \times 10^{-8}$  M, an affinity that is similar to sFv produced against the same antigen from hybridomas. Biodistribution studies demonstrate 1.47% injected dose/g tumor 24 h after injection of  $^{125}$ I-C6.5 into scid mice bearing SK-OV-3 tumors. Tumor:normal organ ratios range from 8.9:1 for kidney to 283:1 for muscle. **Conclusions:** These results are the first in vivo biodistribution studies using an sFv isolated from a non-immune human repertoire and confirm the specificity of sFv produced in this manner. The use of phage display to

**Abbreviations:** sFv, single-chain Fv; IgG, immunoglobulin G; V<sub>H</sub>, immunoglobulin heavy chain variable region; V<sub>L</sub>, immunoglobulin light chain variable region; ECD, extracellular domain; PBS, phosphate-buffered saline; IPTG, isopropyl- $\beta$ -D-thiogalactopyranoside; ABTS, 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid); IMAC, immobilized

metal affinity chromatography; HBS, hepes-buffered saline; CT, chloramine T; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; %ID/g, percentage of injected dose per g of tissue; T:O ratio, tumor:normal organ ratio.

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produce C6.5 mutants with higher affinity and slower  $k_{off}$  would permit rigorous evaluation of the role of antibody affinity and binding kinetics in tumor targeting, and could result in the production of a therapeutically useful targeting protein for radioimmunotherapy and other applications.

**Keywords:** c-erbB-2; sFv; Phage antibody library; Immunotherapy

## 1. Introduction

With the exception of a few limited applications [1], antibody-based reagents have failed to live up to their anticipated role as highly specific targeting agents for cancer therapy. This has likely been the result of suboptimal delivery of antibody to tumor, due a number of factors including the physiology of the tumors and the large size of IgG molecules. The development of single-chain Fv (sFv) molecules, which retain the binding specificity of a parent IgG in a 26-kDa molecule, addresses some of these issues [2]. Radiolabelled anti-tumor sFv penetrate deeply into human tumor xenografts in mice and are cleared rapidly from circulation and normal tissue, resulting in highly specific tumor retention by as early as 4 h after administration [3]. sFv have typically been created from the immunoglobulin variable region genes of murine hybridomas and expressed in *E. coli*. Limitations of this approach include potential immunogenicity of murine sFv and the fact that many sFv express poorly, or not at all in *E. coli* [4]. Production of human antibodies by conventional hybridoma technology has proven difficult. Recently, it has proven possible to produce human sFv directly in *E. coli* by expressing large antibody gene repertoires on the surface of bacteriophage, and selecting phage-expressing binding antibodies by affinity chromatography (phage display) (see Ref. [5] and, for a review, Ref. [6]). In this report, we describe the application of this technique to produce a human sFv (C6.5) that binds to c-erbB-2, an oncogene product overexpressed by 30–50% of breast carcinomas and other adenocarcinomas. In vitro affinity and binding kinetics and in vivo pharmacokinetics in tumor-bearing scid mice are described and compared to values previously determined for 741F8 sFv', an sFv molecule produced from a murine IgG [3].

## 2. Materials and methods

### 2.1. Preparation of c-erbB-2 ECD

c-erbB-2 ECD with a Ser-Gly-His<sub>6</sub> C-terminal fusion was expressed from Chinese Hamster Ovary cells and purified by immobilized metal affinity chromatography (IMAC) as previously described [7].

### 2.2. Phage preparation

Phage were prepared from a phagemid library ( $3 \times 10^7$  members) expressing sFv as pIII fusions on the phage surface [5]. The library was created from a repertoire of sFv genes consisting of human heavy and light chain variable region ( $V_H$  and  $V_L$ ) genes isolated from the peripheral blood lymphocytes of unimmunized human volunteers. To rescue phagemid particles from the library, 50 ml of  $2 \times$  TY media containing 100  $\mu$ g/ml ampicillin and 1% glucose were inoculated with  $10^8$  bacteria taken from the frozen library glycerol stock. The culture was grown at 37°C with shaking to an  $A_{600\text{ nm}}$  of 0.8,  $7.0 \times 10^{11}$  colony-forming units of VCS-M13 (Stratagene) added, and incubation continued at 37°C for 1 h without shaking followed by 1 h with shaking. The cells were pelleted by centrifugation at  $4500 \times g$  for 10 min, resuspended in 200 ml of  $2 \times$  TY media containing 100  $\mu$ g/ml ampicillin and 25  $\mu$ g/ml kanamycin and grown overnight at 37°C. Phage particles were purified and concentrated by two polyethylene glycol precipitations and resuspended in PBS (25 mM  $\text{NaH}_2\text{PO}_4$ , 125 mM NaCl, pH 7.0) to approximately  $10^{13}$  transducing units/ml ampicillin resistant clones.

### 2.3. Selection of binding phage antibodies

Phage-expressing sFv which bound c-erbB-2 were selected by panning the phage library on immobilized c-erbB-2 ECD [5]. Briefly, immuno-

tubes (Nunc, Maxisorb) were coated with 2 ml (100  $\mu\text{g/ml}$ ) c-erbB-2 ECD in PBS overnight at 20°C and blocked with 2% milk powder in PBS for 2 h at 37°C. One ml of the phage solution (approximately  $10^{13}$  phage) was added to the tubes and incubated at 20°C with tumbling on an over and under turntable for 2 h. Non-binding phage were eliminated by sequential washing (15 times with PBS containing 0.05% Tween followed by 15 times with PBS). Binding phage were then eluted from the immunotubes by adding 1 ml of 100 mM triethylamine, incubating for 10 min at 20°C, transferring the solution to a new tube, and neutralizing with 0.5 ml 1 M Tris-HCl, pH 7.4. Half of the eluted phage solution was used to infect 10 ml of *E. coli* TG1 [8] grown to an  $A_{600\text{ nm}}$  of 0.8–0.9. After incubation for 30 min at 37°C, bacteria were plated on TYE plates containing 100  $\mu\text{g/ml}$  ampicillin and 1% glucose and grown overnight at 37°C. Phage were rescued and concentrated as described above and used for the next selection round. The selection process was repeated for a total of five rounds.

#### 2.4. Screening for binders

After each round of selection, 10 ml of *E. coli* HB2151 [9] ( $A_{600\text{ nm}} \sim 0.8$ ) were infected with 100  $\mu\text{l}$  of the phage eluate in order to prepare soluble sFv. In this strain, the amber codon between the sFv gene and gene III is read as a stop codon and native soluble sFv secreted into the periplasm and media [10]. Single ampicillin-resistant colonies were used to inoculate microtitre plate wells containing 150  $\mu\text{l}$  of 2  $\times$  TY containing 100  $\mu\text{g/ml}$  ampicillin and 0.1% glucose. The bacteria were grown to an  $A_{600\text{ nm}} \sim 1.0$ , and sFv expression induced by the addition of IPTG to a final concentration of 1 mM [11]. Bacteria were grown overnight at 30°C, the cells removed by centrifugation, and the supernatant containing sFv used directly.

To screen for binding, 96-well microtitre plates (Falcon 3912) were coated overnight at 4°C with 10  $\mu\text{g/ml}$  c-erbB-2 ECD in PBS, blocked for 2 h at 37°C with 2% milk powder in PBS, and incubated for 1.5 h at 20°C with 50  $\mu\text{l}$  of the *E. coli* supernatant containing sFv. Binding of soluble sFv to antigen was detected with a mouse monoclonal

antibody (9E10) which recognizes the C-terminal *myc* peptide tag [12] and peroxidase-conjugated anti-mouse Fc antibody (Sigma) using ABTS as substrate [13]. The reaction was stopped after 30 min with NaF (3.2 mg/ml) and the  $A_{405\text{ nm}}$  measured. Unique clones were identified by PCR fingerprinting [5] and DNA sequencing. The specificity of each unique sFv was determined by ELISA performed as described above with wells coated with 10  $\mu\text{g/ml}$  of bovine serum albumin, hen egg white lysozyme, bovine glutamyltranspeptidase, c-erbB-2 ECD, VCS M13 ( $3.5 \times 10^{12}/\text{ml}$ ) and casein (0.5%). For ELISA with biotinylated c-erbB-2 ECD, microtitre plates (Immunolon 4, Dynatech) were coated with 50  $\mu\text{l}$  Immunopure avidin (Pierce; 10  $\mu\text{g/ml}$  in PBS) overnight at 4°C, blocked with 1% bovine serum albumin in PBS for 1 h at 37°C and incubated with 50  $\mu\text{l}$  biotinylated c-erbB-2 extracellular domain (5  $\mu\text{g/ml}$ ) for 30 min at 20°C. To prepare biotinylated antigen, 0.2 ml c-erbB-2 ECD (1 mg/ml in PBS) was incubated with 0.5 mM NHS-LC-biotin (Pierce) overnight at 4°C and then purified on a presto desalting column (Pierce).

#### 2.5. Subcloning, expression and purification

To facilitate purification, the C6.5 sFv gene was subcloned into the expression vector pUC119Sfi1/Not1Hismyc [14] which results in the addition of a hexa-histidine tag at the C-terminal end of the sFv. Briefly, pHEN-1 vector DNA containing the C6.5 sFv DNA was prepared by alkaline lysis miniprep, digested with *NcoI* and *NotI*, and the sFv DNA purified on a 1.5% agarose gel. C6.5 sFv DNA was ligated into pUC119Sfi1/Not1Hismyc digested with *NcoI* and *NotI* and the ligation mixture used to transform electrocompetent *E. coli* HB2151. For expression, 200 ml of 2  $\times$  TY media containing 100  $\mu\text{g/ml}$  ampicillin and 0.1% glucose was inoculated with *E. coli* HB2151 harboring the C6.5 gene in pUC119Sfi1/Not1Hismyc. The culture was grown at 37°C to an  $A_{600\text{ nm}}$  of 0.8, soluble sFv expression induced by the addition of IPTG to a final concentration of 1 mM, and the culture grown at 30°C in a shaker flask overnight. sFv was harvested from the periplasm using the following protocol. Cells were harvested by centrifugation at  $4000 \times g$  for 15 min, resuspended in

10 ml of ice-cold 30 mM Tris-HCl, pH 8.0, 1 mM EDTA, 20% sucrose, and incubated on ice for 20 min. The bacteria were pelleted by centrifugation at  $6000 \times g$  for 15 min. and the 'periplasmic fraction' cleared by centrifugation at  $30\,000 \times g$  for 20 min. The supernatant was dialyzed overnight at  $4^\circ\text{C}$  against 8 L of IMAC loading buffer (50 mM sodium phosphate, pH 7.5, 500 mM NaCl, 20 mM imidazole) and then filtered through a  $0.2\text{-}\mu\text{m}$  filter.

sFv was purified by IMAC. All steps were performed at  $4^\circ\text{C}$  on a Perceptive Biosystems BIOCAD Sprint. A column containing 2 ml of Ni-NTA resin (Qiagen) was washed with 20 ml IMAC column wash buffer (50 mM sodium phosphate, pH 7.5, 500 mM NaCl, 250 mM imidazole) and 20 ml of IMAC loading buffer. The periplasmic preparation was loaded onto the column by pump and the column washed sequentially with 50 ml IMAC loading buffer and 50 ml IMAC washing buffer (50 mM sodium phosphate, pH 7.5, 500 mM NaCl, 25 mM imidazole). Protein was eluted with 25 ml IMAC elution buffer (50 mM sodium phosphate, pH 7.5, 500 mM NaCl, 100 mM imidazole) and 4-ml fractions collected. Protein was detected by absorbance at 280 nm and sFv typically eluted between fractions 6 and 8. To remove dimeric and aggregated sFv, samples were concentrated to a volume  $< 1$  ml in a Centricon 10 (Amicon) and fractionated on a Superdex 75 column using a running buffer of HBS (10 mM Hepes, 150 mM NaCl, pH 7.4). The purity of the final preparation was evaluated by assaying an aliquot by SDS-PAGE. Protein bands were detected by Coomassie staining. The concentration was determined spectrophotometrically, assuming an  $A_{280\text{ nm}}$  of 1.0 corresponds to an sFv concentration of 0.7 mg/ml.

#### 2.6. Affinity and kinetic measurements

The  $K_d$  of C6.5 and 741F8 sFv' were determined using surface plasmon resonance in a BIAcore (Pharmacia) and by Scatchard analysis. In a BIAcore flow cell, 1400 resonance units (RU) of c-erbB-2 ECD (25  $\mu\text{g}/\text{ml}$  in 10 mM sodium acetate, pH 4.5) were coupled to a CM5 sensor chip [15]. Association and dissociation of C6.5 and 741F8 sFv' (100–600 nM) were measured under con-

tinuous flow of 5  $\mu\text{l}/\text{min}$ .  $k_{\text{on}}$  was determined from a plot of  $(\ln(dR/dt))/t$  vs. concentration [16].  $k_{\text{off}}$  was determined from the dissociation part of the sensorgram at the highest concentration of sFv analyzed [15]. The  $K_d$  of C6.5 was also determined by Scatchard analysis [17]. All assays were performed in triplicate. Briefly, 50  $\mu\text{g}$  of radioiodinated sFv was added to  $5 \times 10^6$  SK-OV-3 cells in the presence of increasing concentrations of unlabelled sFv from the same preparation. After a 30-min incubation at  $20^\circ\text{C}$ , the samples were washed with PBS at  $4^\circ\text{C}$  and centrifuged at  $500 \times g$ . The amount of labelled sFv bound to the cells was determined by counting the pellets in a gamma counter and the  $K_a$  and  $K_d$  were calculated using the EBDA program (V 2.0, G.A. McPherson, 1983).

#### 2.7. Radiolabelling

The C6.5 sFv was labelled with radioiodine using the CT method [18]. Briefly, 1.0 mg of protein was combined with  $^{125}\text{I}$  (14–17 mCi/mg) (Amersham, Arlington Heights, IL), or  $^{131}\text{I}$  (9.25 mCi/mg) (DuPont NEN, Wilmington, DE) at an iodine to protein ratio of 1:10. Ten  $\mu\text{g}$  of CT (Sigma, St. Louis, MO) was added per 100  $\mu\text{g}$  of protein and the resulting mixture was incubated for 3 min at room temperature. The reaction was quenched by the addition of 10  $\mu\text{g}$  of sodium metabisulfite (Sigma) per 100  $\mu\text{g}$  of protein. Unincorporated radioiodine was separated from the labelled protein by gel filtration using the G-50-80 centrifuged-column method [3]. The final specific activity of the CT labelling was 1.4 mCi/mg for the  $^{131}\text{I}$ -C6.5 sFv and typically about 1.0 mCi/mg for the  $^{125}\text{I}$ -C6.5 sFv.

#### 2.8. Quality control

The quality of the radiopharmaceuticals was evaluated by HPLC, SDS-PAGE, and a live cell binding assay as previously described [3]. The HPLC elution profiles from a Spherogel TSK-3000 molecular sieving column consistently demonstrated that greater than 99% of the radioactivity was associated with the protein peak. Greater than 98% of the non-reduced  $^{125}\text{I}$ -C6.5 sFv preparations migrated on SDS-PAGE as approximately 26  $K_d$  proteins, while the remaining

activity migrated as a dimer. The immunoreactivity of the radiopharmaceuticals was determined in a live cell binding assay utilizing c-erbB-2 overexpressing SK-OV-3 cells (#HTB 77; American Type Culture Collection, Rockville, MD) and c-erbB-2 negative CEM cells (#119; American Type Culture Collection) [3]. Live cell binding assays revealed 49% of the activity associated with the positive cell pellet and less than 3% bound to the negative control cells; these results were lower than those typically seen with 741F8 sFv (60–80% bound) [3].

### 2.9. Cell surface dissociation studies

Cell surface retention of biotinylated forms of the sFv molecules were measured by incubating 2  $\mu\text{g}$  of either sFv with  $2 \times 10^6$  SK-BR-3 cells (#HTB 30; American Type Culture Collection) in triplicate in 20 ml of FACS buffer, with 0.01% azide for 15 min at 4°C. The cells were washed twice with FACS buffer (4°C) and resuspended in 2 ml of FACS buffer; 0.5 ml of the cell suspension were removed and placed in three separate tubes for incubations under differing conditions; 0 min at 4°C, 15 min at 37°C, and 30 min at 37°C. After the incubations, the cells were centrifuged at  $500 \times g$ , the supernatants were removed, the cell pellets were washed twice (4°C) and the degree of retention of sFv on the cell surface at 37°C (for 15 or 30 min) was compared to retention at 0 min at 4°C.

### 2.10. Biodistribution and radioimmunoimaging studies

Four- to six-week-old C.B17/Icr-scid mice were obtained from the Fox Chase Cancer Center Laboratory Animal Facility. SK-OV-3 cells ( $2.5 \times 10^6$ ) in log phase were implanted s.c. on the abdomens of the mice. After about 7 weeks the tumors had achieved sizes of 100–200 mg and Lugol's solution was placed in the drinking water to block thyroid accumulation of radioiodine. Three days later, biodistribution studies were initiated.  $^{125}\text{I}$ -C6.5 sFv was diluted in PBS to a concentration of 0.2 mg/ml and each mouse was given 100  $\mu\text{l}$ , containing 20  $\mu\text{g}$  of radiopharmaceutical, by tail vein injection. Total injected doses were determined by counting each animal on a Series 30 multichannel analyzer/probe system (probe model

#2007, Canaberra, Meridian, CT). Blood samples and whole body counts of the mice were obtained at regular intervals. Groups of eight mice were sacrificed at 24 h after injection and the tumors and organs removed, weighed and counted in a gamma counter to determine the %ID/g [3,19]. The mean and standard error of the mean (SEM) for each group of data were calculated, and T:O ratios determined. Significance levels were determined using Student's *t*-test.

For the radioimmunoimaging studies, tumor-bearing scid mice were injected with 100  $\mu\text{g}$  (100  $\mu\text{l}$ ) of  $^{131}\text{I}$ -C6.5. At 24 h after injection, the mice were euthanized by asphyxiation with  $\text{CO}_2$  and images were acquired on a Prism 2000XP gamma camera (Picker, Highland Heights, OH 44142). Preset acquisitions of 100k counts were used.

## 3. Results

After four rounds of selection, 9/190 clones analyzed by ELISA expressed sFv which bound c-erbB-2 ECD (ELISA signals greater than 0.4, six-times higher than background). After five rounds of selection, 33/190 clones expressed c-erbB-2 binding sFv. PCR fingerprinting of the 42 positive clones identified two unique restriction patterns, and DNA sequencing of six clones from each pattern revealed two unique human sFv sequences, C4.1 and C6.5 (Table 1). The  $V_H$  gene of C6.5 is from the human  $V_H5$  gene family, and the  $V_L$  gene from the human  $V_L1$  family (Table 1). The  $V_L$  gene appears to be derived from two different germline genes (HUMLV122 and DPL 5) suggesting the occurrence of PCR crossover (Table 1). The  $V_H$  gene of C4.1 is from the human  $V_H3$  family, and the  $V_L$  gene from the human  $V_L3$  family (Table 1). C4.1 and C6.5 both bound c-erbB-2 specifically, as determined by ELISA against the relevant antigen and a panel of irrelevant antigens. However, when biotinylated c-erbB-2 ECD was bound to avidin-coated plates and used in ELISA assays, the signal obtained with C6.5 was six-times higher than observed when c-erbB-2 ECD was absorbed to polystyrene (1.5 vs. 0.25). In contrast, C4.1 was not capable of binding to biotinylated c-erbB-2 ECD captured on avidin microtitre plates. Additionally, biotinylated and iodinated C6.5, but

Table 1  
Deduced amino acid sequence of C4.1 and C6.5 heavy and light chain. Sequences are aligned to the most homologous human germline gene. Dashes indicate sequence identity, GL = germline gene sequence. DP58 and DP73 [22], IGLV3S1 [23], HUMLV122 AND DPL 5 [24]

Heavy Chains	Framework 1	CDR1	Framework 2	CDR2	Framework 3	CDR3	Framework 4
C4.1	QVQLVESGGGLVQPGGSLRLSCAAAGFTFS	SYEMN	WVRQAPGKLEWIS	YISSSGSTIYYADSVKIG	RFTISRDNANKNSLIYQMSLRAEDTAVYICAR	DLGGYSYGVYGLDY	WGQGTFLVTVSS
DP58	E-----	-----	-----	-----	-----	-----	-----
C6.5	QVQLLQSGAEIKKPKGESLKIICKGSGYSFT	SYWIA	WRQMPGKLEWIG	LIYPGDSITKYSPFQG	QVTISVDKSVSTAYLQMSLKPDSAVYFCAR	HDVGYCSSNCAKWFPEYFQH	WGQGTFLVTVSS
DP73	E---V---V-----	---G	---W---	I-----R-----	---A---I-----	-----	-----
Light chains	Framework 1	CDR1	Framework 2	CDR2	Framework 3	CDR3	Framework 4
C4.1	SELTQDPAPVAVLGGTVRITC	QGDLSIRSYAS	WYQQKRPQAPVLIYI	GKNNRPS	GIPDRFSGSSSGNIASLITITGAQAEDEADYYC	NSRDSSGNPYWV	FGGSKTKVTVLIG
IGLV3S1	-S-----	-----	-----	-----	-----T-----	-----H V-----	-----
C6.5	QSVLTQPPSVSNAAPQKVTISC	SGSSSNIGNNYVS	WYQQLPGTAPKLLIY	GHTNRPA	GVPDRFSGSKGTSASLAISGRSEDEADYYC	AAMDLSLG WV	FGGSKTKVTVLIG
HUMLV122	-----	-----	-----	DNKK--S	-----T-G-T-LQGTG	GT--S--A	-----
DPL5	-----A-GT--R-----	-----S---Y	-----	RNNQ--S	-----L-----	-----	-----

Table 2  
Characterization of anti-c-erbB-2 sFv species

	741F8	C6.5
$K_d$ (BIAcore)	$2.6 \times 10^{-8}$ M	$1.6 \times 10^{-8}$ M
$K_d$ (Scatchard)	$5.4 \times 10^{-8}$ M	$2.1 \times 10^{-8}$ M
$k_{on}$ (BIAcore)	$2.4 \times 10^5$ $M^{-1}s^{-1}$	$4.0 \times 10^5$ $M^{-1}s^{-1}$
$k_{off}$ (BIAcore)	$6.4 \times 10^{-3} s^{-1}$	$6.3 \times 10^{-3} s^{-1}$
% associated with cell surface at 15 min	32.7	60.6
% associated with cell surface at 30 min	8.6	22.2
%ID/g Tumor	0.8	1.0
T:Blood	14.7	22.9
T:Kidney	2.8	5.6 <sup>a</sup>
T:Liver	14.2	22.3
T:Spleen	10.3	34.1
T:Intestine	25.0	29.7
T:Lung	9.4	15.8
T:Stomach	8.9	11.1
T:Muscle	78.8	158.7
T:Bone	30.0	102.7

Characteristics of the murine anti-c-erbB-2 sFv, 741F8, and the human sFv C6.5 are compared. The affinity and dissociation constants were determined by Scatchard plot analysis, unless otherwise stated. Dissociation from c-erbB-2-positive (SK-OV-3) cells was measured in an in vitro live cell assay. The percentage of injected dose per gram (%ID/g) tumor (T) and tumor to organ ratios were determined in biodistribution studies performed in separate groups of scid mice ( $n = 10-14$ ) bearing SK-OV-3 tumors overexpressing c-erbB-2. SEM are < 35% of the associated values.

<sup>a</sup>Significantly improved, ( $P < 0.05$ ) compared to 741F8 sFv.

not C4.1, bound SK-BR-3 cells overexpressing c-erbB-2. These results indicate that C6.5 binds the native c-erbB-2 expressed on cells, but C4 binds a denatured epitope that appears when the antigen is absorbed to polystyrene.

C6.5 was purified in yields of 10 mg/l of *E. coli* grown in shake flasks and gel filtration analysis indicated a single peak of approximately 27  $K_d$ . The  $K_d$  of purified C6.5 was determined using both surface plasmon resonance in a BIAcore and by Scatchard analysis. The  $K_d$  determined by BIAcore ( $1.6 \times 10^{-8}$  M) agreed closely to the value determined by Scatchard ( $2.0 \times 10^{-8}$  M) (Table 2). Kinetic analysis by BIAcore indicated that C6.5 had a rapid on-rate ( $k_{on}$   $4.0 \times 10^5 M^{-1} s^{-1}$ ) and a rapid off-rate ( $k_{off}$   $6.3 \times 10^{-3} s^{-1}$ )

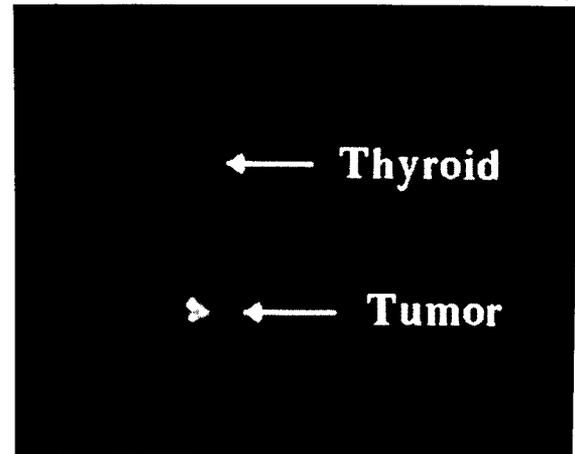


Fig. 1. Radioimmunoimaging of subcutaneous SK-OV-3 tumors in C.B17/ICR-scid mice by  $^{131}I$ -C6.5. Gamma camera images were obtained at 24 h after the i.v. administration of 100  $\mu$ g (140  $\mu$ Ci) of C6.5. Image acquisition was terminated when 100 000 counts were acquired.

(Table 2). Cell retention assay confirmed that C6.5 dissociated rapidly from the cell surface (Table 2).

After injection of  $^{125}I$ -C6.5 into scid mice bearing SK-OV-3 tumors, 1.47% %ID/g of tumor was retained after 24 h (Table 2). Tumor:normal organ values ranged from 8.9 (tumor:kidney) to 283 (tumor:muscle). These values were higher than values observed for 741F8 sFv produced from a murine monoclonal antibody ( $K_d = 2.6 \times 10^{-8}$  M). The high T:O ratios resulted in the highly specific visualization of the tumor by gamma scintigraphy using  $^{131}I$ -labelled C6.5 (Fig. 1).

#### 4. Discussion

We have isolated a human sFv from a non-immune phage antibody library which binds specifically to c-erbB-2 in vitro and in vivo. These results are the first in vivo biodistribution studies using an antibody fragment isolated from a non-immune human repertoire, and confirm the specificity of sFv produced in this manner. C6.5 expresses at high level as native protein in *E. coli*, is easily purified in high yield in two steps, and has

an affinity that is similar to sFv produced from hybridomas [3]. The results illustrate potential advantages of this approach compared to producing sFvs from hybridomas. First, the antibodies are entirely human in sequence, and are less likely to be immunogenic than murine sFv. Second, the approach is significantly faster. A single library provides antibodies against most antigens and selections take only 2 weeks to perform. For each hybridoma, however, the  $V_H$  and  $V_L$  genes have to be successfully isolated and cloned as an sFv DNA construct, a relatively time-consuming process. Once the genes have been successfully cloned, expression levels of different sFv in bacteria vary considerably, and in many instances are too low to produce adequate quantities of protein for characterization and in vivo studies [4]. Even in exceptional cases where very high sFv refolding yields are obtained [20], the final product is a mixture of non-native and native sFv, which are best separated by affinity chromatography. In contrast, sFv produced using phage display are typically expressed at high level in *E. coli* as native protein [5], and are readily purified by a non-functional isolation such as IMAC.

One of the two sFv isolated bound c-erbB-2 immobilized on polystyrene, but not biotinylated c-erbB-2 or c-erbB-2 expressing cells. The result suggests that adsorption partially denatures the protein, exposing epitopes that do not exist in solution. Likewise, C6.5 bound biotinylated c-erbB-2 with higher ELISA signal than adsorbed c-erbB-2 and also bound c-erbB-2 expressing cells. Thus, selections performed in solution using biotinylated antigen should optimize the probability that selected sFv will recognize native antigen.

Although C6.5 has an affinity comparable to sFv derived from hybridomas, the  $k_{off}$  is relatively rapid, less than 30% of C6.5 remains bound to cell surface c-erbB-2 after 15 min. It should be possible to significantly reduce the  $k_{off}$ , and decrease the  $K_d$ , by creating and selecting mutant C6.5 phage antibody libraries. We have used this approach to decrease the  $K_d$  of a hapten binding human sFv 320-fold, while reducing the  $k_{off}$  greater than 100-fold [21]. Production of C6.5 mutants with higher affinity and slower  $k_{off}$  would permit rigorous evaluation of the role of antibody affinity and

binding kinetics in tumor targeting, and could result in the production of a therapeutically useful targeting protein for radioimmunotherapy and other applications.

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Appendix 2

Schier R, Bye J, Apell G, McCall A, Adams GP, Malmqvist M, Weiner LM, Marks JD. Isolation of high affinity human anti-c-erbB-2 single chain Fv using affinity driven selection. *J. Mol. Biol.*, 255: 28-43, 1996.

## Isolation of High-affinity Monomeric Human Anti-*c-erbB-2* Single chain Fv Using Affinity-driven Selection

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The use of antibodies to target tumor antigens has had limited success, partially due to the large size of IgG molecules, difficulties in constructing smaller single chain Fv (scFv) antibody fragments, and immunogenicity of murine antibodies. These limitations can be overcome by selecting human scFv directly from non-immune or semi-synthetic phage antibody libraries; however, the affinities are typically too low for therapeutic application. For hapten antigens, higher-affinity scFv can be isolated from phage antibody libraries where the  $V_H$  and  $V_L$  genes of a binding scFv are replaced with repertoires of V genes (chain shuffling). The applicability of this approach to protein binding scFv is unknown. For this work, chain shuffling was used to increase the affinity of a non-immune human scFv, which binds the glycoprotein tumor antigen *c-erbB-2* with an affinity of  $1.6 \times 10^{-8}$  M. The affinity of the parental scFv was increased sixfold ( $K_d = 2.5 \times 10^{-9}$  M) by light-chain shuffling and fivefold ( $K_d = 3.1 \times 10^{-9}$  M) by heavy-chain shuffling, values comparable to those for antibodies against the same antigen produced by hybridomas. When selections were performed on antigen immobilized on polystyrene, spontaneously dimerizing scFv were isolated, the best of which had only a slightly lower  $K_d$  than wild type ( $K_d = 1.1 \times 10^{-9}$  M). These scFv dimerize on phage and are preferentially selected as a result of increased avidity. Compared to scFv which formed only monomer, dimerizing scFv had mutations located at the  $V_H$ - $V_L$  interface, suggesting that  $V_H$ - $V_L$  complementarity determines the extent of dimerization. Higher-affinity monomeric scFv were only obtained by selecting in solution using limiting concentrations of biotinylated antigen, followed by screening mutant scFv from bacterial periplasm by  $k_{off}$  in a BIAcore. Using the proper selection and screening conditions, protein binding human scFv with affinities comparable to murine hybridomas can be produced without immunization.

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**Keywords:** *c-erbB-2*; single chain Fv; phage antibody libraries; affinity maturation; chain shuffling

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Abbreviations used: AMP, ampicillin; *c-erbB-2* ECD, extracellular domain of *c-erbB-2*; CDR, complementarity determining region; ELISA, enzyme-linked immunosorbent assay; FR, framework region; Glu, glucose; HBS, Hepes-buffered saline (10 mM Hepes, 150 mM NaCl, pH 7.4); IMAC, immobilized metal affinity chromatography; IPTG, isopropyl- $\beta$ -D-thiogalactopyranoside; KAN, kanamycin;  $k_{on}$ , association rate constant;  $k_{off}$ , dissociation rate constant; MPBS, skimmed milk powder in PBS; PBS, phosphate buffered saline (25 mM  $\text{NaH}_2\text{PO}_4$ , 125 M NaCl, pH 7.0); PCR, polymerase chain reaction; RU,

### Introduction

Despite the demonstration of tumor-specific and tumor-associated antigens, the use of monoclonal antibodies for therapy of cancers has not yielded

resonance units; scFv, single chain Fv fragments; TPBS, 0.05% (v/v) Tween 20 in PBS; t.u., transducing units;  $V_\kappa$ , immunoglobulin kappa light chain variable region;  $V_\lambda$ , immunoglobulin lambda light chain variable region;  $V_L$ , immunoglobulin light chain variable region;  $V_H$ , immunoglobulin heavy chain variable region.

consistent beneficial responses (reviewed by Riethmuller *et al.*, 1993). The disappointing results can be attributed partially to limitations of monoclonal IgG antibodies and limitations of the hybridoma technology used to generate them. IgG are large molecules (150 kDa) that diffuse slowly into tumors (Clauss & Jain, 1990) and are slowly cleared from the circulation, resulting in poor tumor:normal organ ratios (Sharkey *et al.*, 1990). Smaller single-chain Fv antibody fragments (scFv, 25 kDa; Bird *et al.*, 1988; Huston *et al.*, 1988) penetrate tumors better than IgG (Yokota *et al.*, 1992), are cleared more rapidly from the circulation, and provide greater targeting specificity (Colcher *et al.*, 1988; Milenic *et al.*, 1991; Adams *et al.*, 1993). scFv are monovalent, however, and dissociate from tumor antigen faster than divalent IgG molecules, which exhibit a higher apparent affinity due to avidity (Crothers & Metzger, 1972). This feature, combined with rapid clearance from the blood, results in significantly lower quantitative retention of scFv in the tumor. This limitation could be overcome by creating higher-affinity scFv with slower dissociation rate constants ( $k_{off}$ ).

Until recently, scFv have proven relatively difficult to produce and engineer. Traditional approaches have involved cloning the rearranged immunoglobulin heavy ( $V_H$ ) and light chain ( $V_L$ ) variable region genes from murine hybridomas into bacterial expression vectors. The scFv is then expressed intracellularly and refolded from inclusion bodies, or secreted into the periplasm as native scFv protein. This approach has a number of limitations. For each hybridoma, the rearranged  $V_H$  and  $V_L$  genes have to be successfully cloned and assembled as an scFv gene construct, which is a relatively time-consuming process. Once cloned, scFv expression levels vary considerably, and in many instances are too low to produce adequate quantities of scFv for further characterization (Knappik *et al.*, 1993). This is particularly true when scFv have to be refolded from inclusion bodies (Huston *et al.*, 1991). Even in exceptional cases where refolding yields are high, the final product is a mixture of non-native and native scFv, which are best separated by affinity chromatography (Huston *et al.*, 1991). Finally, scFv derived from hybridomas are murine in sequence and may be immunogenic when administered to humans.

The above limitations can be overcome by producing human scFv directly in bacteria without immunization. Antigen-specific scFv are selected from non-immune (Marks *et al.*, 1991, 1993; Griffiths *et al.*, 1993) or semi-synthetic (Hoogenboom & Winter, 1992; Nissim *et al.*, 1994) human scFv gene repertoires displayed on the surface of bacteriophage (McCafferty *et al.*, 1990; Hoogenboom *et al.*, 1991). scFv produced in this manner are almost invariably expressed at high level in *Escherichia coli* as native protein (Marks *et al.*, 1991; Schier *et al.*, 1995) and are specific for the antigen used for selection. Using this approach, we isolated a human scFv (C6.5) from a non-immune phage antibody library (Marks *et al.*, 1991) that binds specifically to the extracellular domain (ECD) of the tumor antigen *c-erbB-2*

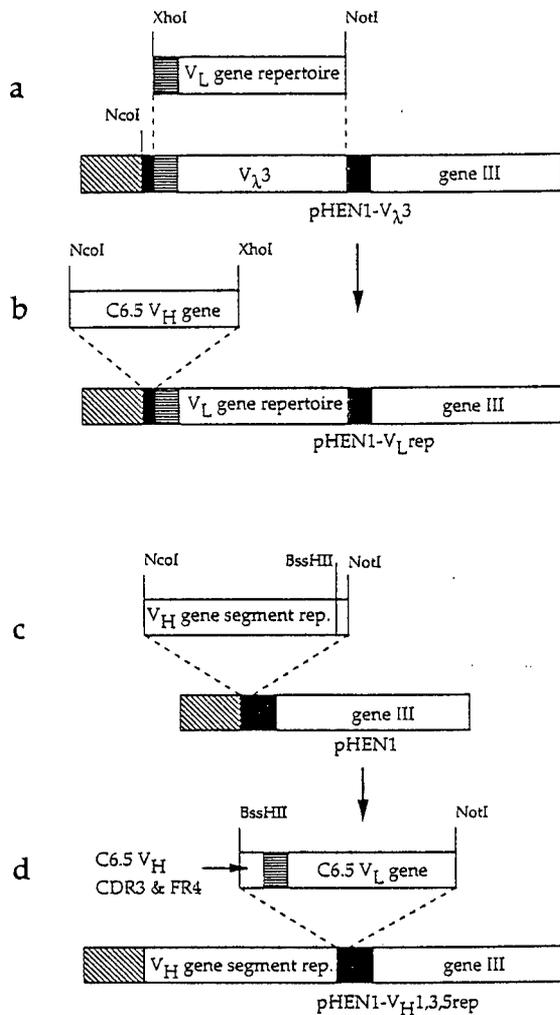
(McCartney *et al.*, 1995) with a  $K_d$  of  $1.6 \times 10^{-8}$  M and  $k_{off}$  of  $6.3 \times 10^{-3} \text{ s}^{-1}$  (Schier *et al.*, 1995). Biodistribution studies in scid mice demonstrated high tumor:normal organ ratios and excellent tumor visualization (Schier *et al.*, 1995); however, quantitative delivery of scFv to tumor is inadequate to provide therapeutic dosimetry. Greater delivery should be possible with higher-affinity scFv. Affinity can be increased by creating mutant phage antibody libraries and selecting higher-affinity antibody fragments (Marks *et al.*, 1992; Hawkins *et al.*, 1992, 1993; Riechmann & Weill, 1993; Barbas *et al.*, 1994; Deng *et al.*, 1994). One approach to create mutant scFv gene repertoires is to replace the  $V_H$  or  $V_L$  gene with a V gene repertoire (chain shuffling) (Clackson *et al.*, 1991; Kang *et al.*, 1991). The approach has been used successfully to increase the affinity of a non-immune human scFv that bound the hapten phenylloxazolone 300-fold from  $3.0 \times 10^{-7}$  M to  $1.0 \times 10^{-9}$  M by sequentially shuffling the rearranged  $V_L$  gene and the  $V_H$  gene segment (the wild-type  $V_H$  third complementarity determining region (CDR) was retained) (Marks *et al.*, 1992). Most relevant antigens, however, are proteins, and it is unclear whether chain shuffling would be effective to increase the affinity of protein binding antibody fragments. Shuffling immune rearranged  $V_H$  and  $V_L$  genes of gp120 binding Fabs resulted in Fabs of "similar apparent binding constants" (Collet *et al.*, 1992; Barbas *et al.*, 1993). Compared to antibodies that bind haptens, there are a greater number of contacts between protein and antibody with a greater surface area buried upon binding. Thus the chances of disrupting multiple favorable contacts by shuffling is greater, but could be compensated by the loss of unfavorable contacts, or the generation of new contacts.

For this study, we investigated the utility of chain shuffling to increase the affinity of C6.5. Universal phage display vectors were created that contained either a human  $V_H$  gene segment repertoire or a rearranged  $V_L$  gene repertoire. These vectors permit light-chain shuffling by subcloning the rearranged  $V_H$  gene from an antigen binding scFv, and heavy-chain shuffling by subcloning the rearranged  $V_L$  gene, linker, and  $V_H$  CDR3. The shuffling vectors were used to increase the affinity of C6.5 for *c-erbB-2* sixfold, to  $2.5 \times 10^{-9}$  M, comparable to the affinity of antibodies to the same antigen produced from hybridomas. Higher-affinity scFv was retained on the surface of *c-erbB-2* expressing cells three times longer than the parental scFv. Successful isolation of higher-affinity scFv required the use of limiting antigen concentration and a BIAcore-based screening technique.

## Results

### Construction of shuffled phage antibody libraries

For light-chain shuffling, rearranged human  $V_\lambda$  and  $V_\lambda$  gene repertoires were cloned into the phage



**Figure 1.** Construction of C6.5 chain shuffled libraries. **a**, Construction of a human light-chain library for light-chain shuffling. PCR was used to create a human light-chain gene repertoire with DNA encoding the single chain linker (G<sub>4</sub>S)<sub>3</sub> spliced to the 5' end (horizontally hatched box). The V<sub>L</sub> gene repertoire–single chain linker was cloned as an XhoI-NotI fragment into pHEN1-V<sub>λ</sub>3 (Hoogenboom *et al.*, 1992) to create the library vector pHEN1-V<sub>L</sub>rep. **b**, Construction of C6.5 light-chain shuffled library. The rearranged C6.5 V<sub>H</sub> gene was cloned as an NcoI-XhoI fragment into pHEN1-V<sub>L</sub>rep. **c**, Construction of human heavy-chain libraries for light-chain shuffling. PCR was used to create human V<sub>H</sub>1, V<sub>H</sub>3, and V<sub>H</sub>5 family gene segment repertoires (FR1-FR3, excluding CDR3) containing a BssHIII site at the end of FR3. The V<sub>H</sub> gene segment repertoires were cloned as NcoI-NotI fragments into pHEN1 (Hoogenboom *et al.*, 1991) to create the library vector pHEN1-V<sub>H</sub>1rep, pHEN1-V<sub>H</sub>3rep, or pHEN1-V<sub>H</sub>5rep. **d**, Construction of C6.5 heavy chain shuffled libraries. The C6.5 V<sub>H</sub> CDR3 gene, single chain linker gene, and light chain gene were cloned as a BssHIII-NotI fragment into pHEN1-V<sub>H</sub>1.3.5rep, pHEN1-V<sub>H</sub>3rep, or pHEN1-V<sub>H</sub>5rep. (▨) pelB leader sequence; (■) multiple cloning site polylinker; (▩) myc peptide tag; (▧) (G<sub>4</sub>S)<sub>3</sub> single chain linker.

display vector pHEN1-V<sub>λ</sub>3 (Hoogenboom & Winter, 1992) to create a  $4.5 \times 10^9$  member library (pHEN1-V<sub>L</sub>rep, Figure 1a). The resulting library contains DNA encoding the single-chain linker sequence

(G<sub>4</sub>S)<sub>3</sub>, and cloning sites for inserting the rearranged V<sub>H</sub> gene from a binding scFv as an NcoI-XhoI fragment (Figure 1b). Polymerase chain reaction (PCR) screening of pHEN1-V<sub>L</sub>rep revealed that 95% of clones analyzed had a full-length insert and a diverse BstNI restriction pattern. To shuffle the light chain of C6.5, the rearranged C6.5 V<sub>H</sub> gene was cloned into pHEN1-V<sub>L</sub>rep as an NcoI-XhoI fragment (Figure 1b). After transformation, a library of  $2.0 \times 10^6$  clones was obtained. PCR screening revealed that 100% of clones analyzed had full-length insert and a diverse BstNI restriction pattern. Prior to selection, 0/92 clones selected at random expressed scFv that bound *c-erbB-2*.

Since we were interested in shuffling the V genes of scFv derived from non-immune libraries, heavy chain shuffling libraries were constructed so that only the V<sub>H</sub> gene segment (excluding the V<sub>H</sub> CDR3) was shuffled. The rationale is that V<sub>H</sub> CDR3 results from splicing of three gene segments (V<sub>H</sub>, D, and J), is the most genetically diverse part of the rearranged V<sub>H</sub> gene, and in non-immune repertoires it is unlikely that many similar V<sub>H</sub> CDR3s exist. Since V<sub>H</sub> CDR3 contributes a disproportionate number of amino acid residues that contact antigen, shuffling the rearranged V<sub>H</sub> gene would result in a library containing few binding scFv. For heavy-chain shuffling, human V<sub>H</sub> gene segment repertoires (framework 1 (FR1) to FR3) were cloned into the phage display vector pHEN1 (Hoogenboom *et al.*, 1991) (Figure 1c). The resulting library contains a human V<sub>H</sub> gene segment repertoire and cloning sites for inserting the V<sub>H</sub> CDR3, FR4, single chain linker, and rearranged light chain gene from a binding scFv as a BssHIII-NotI fragment (Figure 1d). Three heavy-chain gene repertoires were created (pHEN1-V<sub>H</sub>1rep, pHEN1-V<sub>H</sub>3rep, and pHEN1-V<sub>H</sub>5rep), each enriched for V<sub>H</sub>1, V<sub>H</sub>3, or V<sub>H</sub>5 gene segments by using PCR primers designed to anneal to the consensus sequence of the 3' end of V<sub>H</sub>1, V<sub>H</sub>3, or V<sub>H</sub>5 FR3 (Tomlinson *et al.*, 1992; see Table 1). These primers also introduced a BssHIII site at the end of FR3, without changing the amino acid sequence typically observed at these residues. Libraries were constructed from these three V<sub>H</sub> gene families, since they make up over 95% of the V<sub>H</sub> genes of non-immune scFv (Marks *et al.*, 1992, 1993; Griffiths *et al.*, 1993). Libraries of  $5.0 \times 10^5$  clones for pHEN1-V<sub>H</sub>1rep,  $1.0 \times 10^6$  clones for pHEN1-V<sub>H</sub>3rep and  $1.5 \times 10^6$  clones for pHEN1-V<sub>H</sub>5rep were obtained. Analysis of 50 clones from each library indicated that greater than 80% of the clones had inserts, and the libraries were diverse, as shown by the BstNI restriction pattern (Marks *et al.*, 1991). Three heavy-chain shuffled libraries were made by cloning the C6.5 V<sub>H</sub> CDR3, FR4, linker, and light-chain gene into the previously created V<sub>H</sub>1, V<sub>H</sub>3, or V<sub>H</sub>5 gene segment library vectors using the BssHIII and NotI restriction sites (Figure 1d). After transformation, libraries of  $1.0 \times 10^6$  to  $2.0 \times 10^6$  clones were obtained. PCR screening revealed that 100% of clones analyzed had a full-length insert and a diverse BstNI restriction pattern. Prior to

Table 1. Sequences of primers used

LMB3	5' -CAGGAAACAGCTATGAC-3'
fd-seq1	5' -GAATTTTCTGTATGAGG-3'
pHEN-1seq	5' -CTATGCGGCCCCATTCA-3'
Linkseq	5' -CGATCCGCCACCCGAGAG-3'
PVH1FOR1	5' -TCGCGCGCAGTAATACACGGCCGTGTC-3'
PVH3FOR1	5' -TCGCGCGCAGTAATACACAGCCGTGTCCTC-3'
PVH5FOR1	5' -TCGCGCGCAGTAATACATGGCCGTGTC-3'
PVH1FOR2	5' -GAGTCATTCTCGACTTGGCGCCGCTCGCGCGCAGTAATACACGGCCGTGTC-3'
PVH3FOR2	5' -GAGTCATTCTCGACTTGGCGCCGCTCGCGCGCAGTAATACACAGCCGTGTCCTC-3'
PVH5FOR2	5' -GAGTCATTCTCGACTTGGCGCCGCTCGCGCGCAGTAATACATGGCCGTGTC-3'
PC6VL1BACK	5' -AGCGCCGTGTATTTTGGCGCGGACATGACGTGGGATATTGC-3'
RJH1/2/6Xho	5' -ACCCTGGTCACCGTCTCGAGTGGTGGA-3'
RJH3Xho	5' -ACAATGGTCACCGTCTCGAGTGGTGGA-3'
RJH4/5Xho	5' -ACCCTGGTCACCGTCTCGAGTGGTGGA-3'
PC6VH1	5' -GAGTCATTCTCGTCTCGAGACGGTGACCAGGGTGCC-3'

selection, 20/92 clones selected at random from the  $V_{H5}$  shuffled library expressed scFv that bound *c-erbB-2*. 0/92 clones selected at random from the  $V_{H1}$  or  $V_{H3}$  shuffled library expressed scFv that bound *c-erbB-2*.

#### Isolation and characterization of higher-affinity light-chain shuffled scFv

In a first approach to increase affinity, *c-erbB-2* ECD-coated polystyrene tubes were used for selecting the light-chain shuffled library. Phage were subjected to three rounds of the rescue-selection-infection cycle. One hundred and eighty clones from the second and the third round of selection were analyzed for binding to recombinant *c-erbB-2* ECD by enzyme-linked immunosorbent assay (ELISA). After the third round of selection, greater than 50% of the clones were positive by ELISA (Table 2). Positive clones were ranked by  $IC_{50}$  as determined by competition ELISA. Sixteen scFv with  $IC_{50}$  values less than the  $IC_{50}$  of the parental scFv were sequenced and four unique DNA sequences were identified (Table 3). All four rearranged  $V_{\lambda}$  genes were putatively derived from the same  $V_{\lambda}$  family ( $V_{\lambda}1$ ) and germline

gene segment (DPL5; Williams & Winter, 1993) as C6.5. These clones were purified by immobilized metal affinity chromatography (IMAC) and gel filtration after subcloning into pUC119Sfi/Notmyc-His. Gel filtration analysis of the four purified scFv demonstrated the presence of two species, with size consistent for monomeric and dimeric scFv (see Figure 2 for a representative example). In contrast, the parental scFv existed only as monomer (Figure 2). The affinity of the monomeric scFv fraction was determined by BIAcore by measuring the association rate constant ( $k_{on}$ ) and  $k_{off}$ , and calculating  $K_d$ . Despite their lower  $IC_{50}$  values, three of the four light-chain shuffled scFv did not have a higher affinity for *c-erbB-2* than C6.5 (Table 4). The fourth (C6VLF) had only a slightly lower  $K_d$  ( $1.1 \times 10^{-8}$  M) than C6.5.  $k_{off}$  of the dimeric scFv fraction was three to fourfold slower than the  $k_{off}$  of the monomeric fraction (Table 4), indicating a significant avidity effect for the scFv dimer. Retention of C6VLD monomeric scFv fraction on the surface of SK-OV-3 cells (12% at 30 minutes) was no different than the retention of C6.5 (10% at 30 minutes), consistent with the similarities in  $k_{off}$  for the two scFv.

Table 2. Frequency of binding scFv and percentage of binding scFv with slower  $k_{off}$  than C6.5

Library and method of selection	ELISA Round of selection			scFv with slower $k_{off}$ than C6.5 (parental scFv) (%) Round of selection		
	2	3	4	2	3	4
$V_L$ -shuffling, selected on:						
Antigen-coated immunotubes	41/180	97/180	ND	ND	ND	ND
Soluble antigen (rd 1, 100 nM; rd 2, 50 nM; rd 3 10 nM; rd 4, 1 nM)	74/90	22/90	13/90	ND	0	42
Soluble antigen (rd 1, 40 nM; rd 2, 1 nM; rd 3 0.1 nM; rd 4, 0.01 nM)	ND	65/90	62/90	ND	25	84
$V_H$ -shuffling, selected on:						
Soluble antigen; (rd 1, 100 nM; rd 2, 50nM; rd 3 10 nM; rd 4, 1 nM)	ND	43/90	56/90	ND	0	0
Soluble antigen (rd 1, 40 nM; rd 2, 1 nM; rd 3 0.1 nM; rd 4, 0.01 nM)	ND	90/90	82/90	ND	0	12

Binding was determined by ELISA.  $k_{off}$  was determined by BIAcore on unpurified scFv in bacterial periplasm. rd, round; ND, not determined; nM,  $1 \times 10^{-9}$  M.

Table 3. Deduced protein sequences of light-chain variable region genes of C6.5 and chain shuffled mutants

	Framework 1	CDR1	Framework 2	CDR2	Framework 3	CDR3	Framework 4					
	10	20	30	35	40	50	60	70	80	90	100	
C6.5	QSVLTPPPSVAAPGOKVTISC	SGSSNIGNNIVS	WYQQLPCTAPKLLIY	GHTNREA	GVPDRFSGSKSQTSAISLAIISGFRSEDEADYYC	AAWDDSLSG WW	FGGCTKLIIVLG					
DPL5*	-----A-GT-----R-----	-----S---Y	-----L---Y	-----S---S	-----I-	-----	-----	-----	-----	-----	-----	
<i>Light-chain shuffled mutants selected on polystyrene adsorbed antigen</i>												
C6VLB	-----	-----	-----	SDNQ--S	-----L-	-----H-	-----	-----	-----	-----	-----	
C6VLD	-----	-----	-----TN-----	TNDQ--S	-----LQ-	-----N--M	-----	-----	-----	-----	-----	
C6VLE	-----	-----A-----	-----S--R-----	RNNQ--S	-----V-	-----S--N	-----	-----	-----	-----	-----V-----	
C6VLF	-----	-----M-----	-----F-----	DNNK--S	I--I-----	-----LQ-D	-----	-----	-----	-----	-----	
<i>Light-chain shuffled mutant selected on biotinylated antigen</i>												
C6L1	-----G---W-----	-----	-----	DNNK--S	-----Q-----	-----L-	-----	-----	-----	-----	-----V-----	

CDR, complementarity-determining region; dashes indicate sequence identity. Numbering is according to Kabat *et al.* (1987). Underlined residues are those that form the  $\beta$ -sheet interface that packs on the  $V_H$  domain (Chothia *et al.*, 1985).

\*The C6.5  $V_L$  gene is putatively derived from the DPL5 germline gene (Williams & Winter, 1993).

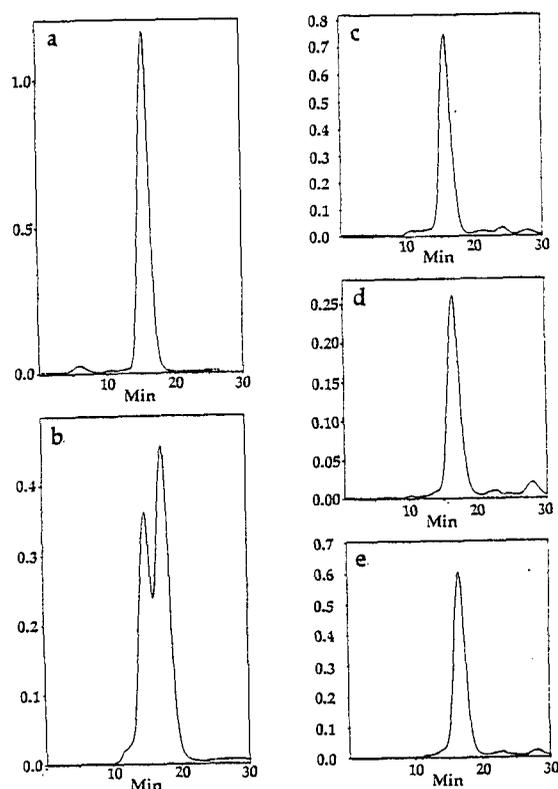


Figure 2. Results of gel filtration analysis of C6.5 scFv and chain shuffled mutants. scFv were purified from bacterial periplasm by immobilized metal affinity chromatography and analyzed by gel filtration on a calibrated Superdex 75 column. a, C6.5 scFv; b, C6VLB scFv; c, C6L1 scFv; d, C6H1; e, C6H2 scFv. scFv selected on *c-erbB-2* immobilized on polystyrene (C6VLB) formed a mixture of monomer and dimer. In contrast, wild-type C6.5 and scFv selected on *c-erbB-2* in solution (C6L1, C6H1, and C6H2) were monomeric.

As a result of these observations, we put forward the hypothesis that selection on immobilized antigen favored the isolation of dimeric scFv that could achieve a higher apparent affinity due to avidity. In addition, determination of  $IC_{50}$  by inhibition ELISA using native scFv in periplasm did not successfully screen for scFv of higher affinity. To avoid the selection of lower-affinity dimeric scFv, subsequent selections were performed in solution by incubating the phage with biotinylated *c-erbB-2* ECD, followed by capture on streptavidin-coated magnetic beads. To select phage on the basis of affinity, the antigen concentration was reduced each round of selection to below the range of the desired scFv  $K_d$  (Hawkins *et al.*, 1992). To screen ELISA positive scFv for improved binding to *c-erbB-2*, we used a BIAcore. Periplasm preparations containing unpurified native scFv could be applied directly to a *c-erbB-2*-coated BIAcore flowcell, and the  $k_{off}$  determined from the dissociation portion of the sensorgram. This permitted ranking the chain shuffled clones by  $k_{off}$ . Moreover, by plotting  $\ln(R_t/R_0)$  versus  $t$ , the presence of multiple  $k_{off}$  could be detected, indicating the presence of mixtures of monomers, dimers, or higher molecular mass scFv.

This strategy of selecting on antigen in solution, followed by BIAcore screening of ELISA positive scFv, was used to isolate higher-affinity chain shuffled mutants.

We reselected the light-chain shuffled library using four rounds of selection on decreasing soluble antigen concentration (100 nM, 50 nM, 10 nM, and 1 nM). In a separate set of experiments, the four rounds of selection were performed using 40 nM, 1 nM, 0.1 nM, and 0.01 nM antigen concentrations. Using the higher set of antigen concentrations for selection, 13/90 clones were positive for *c-erbB-2* binding by ELISA after the fourth round of selection (Table 2). In the BIAcore, 42% of these clones had a slower  $k_{off}$  than the parental scFv (Table 2). Using the lower set of antigen concentrations for selection, more clones were positive for *c-erbB-2* binding by ELISA (62/90) after the fourth round of selection, and 84% had a slower  $k_{off}$  than the parental scFv (Table 2). Sequencing of the  $V_L$  gene of ten of these scFv revealed one unique scFv (C6L1) (Table 3). The  $V_L$  gene of C6L1 was derived from the same germline gene (DPL5; Williams & Winter, 1993) as the parental scFv, but had nine amino acid substitutions. The C6L1 gene was subcloned and the scFv purified by IMAC and gel filtration. C6L1 scFv was monomeric as determined by gel filtration (Figure 2) and had an affinity six times higher than the parental scFv (Table 4). The increased affinity was due to both a faster  $k_{on}$  and a slower  $k_{off}$  (Table 4). The threefold slower  $k_{off}$  correlated with a threefold increase in the retention of scFv on the surface of SK-OV-3 cells (28% at 30 minutes for C6L1 compared to 10% at 30 minutes for C6.5).

#### Isolation and characterization of higher-affinity heavy-chain shuffled scFv

The  $V_{H5}$  heavy-chain shuffled library was subjected to four rounds of selection on decreasing soluble antigen concentration (100 nM, 50 nM, 10 nM, and 1 nM). In a separate set of experiments, the four rounds of selection were performed using 40 nM, 1 nM, 0.1 nM, and 0.01 nM antigen concentrations. Using the higher set of antigen concentrations for selection, 56/90 clones were positive for *c-erbB-2* binding by ELISA after the fourth round of selection (Table 2). None of these clones, however, had a slower  $k_{off}$  than the parental scFv. Using the lower set of antigen concentrations for selection, more clones were positive for *c-erbB-2* binding by ELISA (82/90) after the fourth round of selection, and 12% had a slower  $k_{off}$  than the parental scFv (Table 2). No binders were isolated from either the  $V_{H1}$  or  $V_{H3}$  shuffled libraries. Sequencing of the rearranged  $V_H$  gene of all slower  $k_{off}$  clones revealed two unique scFv, C6H1 and C6H2 (Table 5). The  $V_H$  gene segments of C6H1 and C6H2 were putatively derived from the same germline gene family ( $V_{H5}$ ) and germline gene (DP73; Tomlinson *et al.*, 1992) as the parental scFv but differed by seven and nine amino acids, respectively. C6H1 also had an opal stop codon (TGA) in the heavy-chain CDR1 and

Table 4. Affinities and binding kinetics of c-erbB-2 binding scFv

scFv source and clone name	$K_d$ (M)	$k_{on}$ ( $\times 10^5$ M <sup>-1</sup> s <sup>-1</sup> )	$k_{off}$ ( $\times 10^{-3}$ s <sup>-1</sup> )
<i>Parental</i>			
C6.5	$1.6 \times 10^{-8}$	$4.0 \pm 0.20$	$6.3 \pm 0.06$
<i>Light-chain shuffled library, selected on immobilized antigen</i>			
C6VLB monomer	$3.4 \times 10^{-8}$	$2.9 \pm 0.31$	$10.0 \pm 0.04$
dimer	ND	ND	$2.6 \pm 0.03$
C6VLD monomer	$1.9 \times 10^{-8}$	$3.1 \pm 0.19$	$5.9 \pm 0.05$
dimer	ND	ND	$1.7 \pm 0.06$
C6VLE monomer	$3.3 \times 10^{-8}$	$1.3 \pm 0.13$	$4.3 \pm 0.04$
dimer	ND	ND	$1.5 \pm 0.07$
C6VLF monomer	$1.1 \times 10^{-8}$	$3.7 \pm 0.11$	$4.1 \pm 0.06$
dimer	ND	ND	$1.1 \pm 0.08$
<i>Light-chain shuffled library, selected on soluble antigen</i>			
C6L1	$2.6 \times 10^{-8}$	$7.8 \pm 0.17$	$2.0 \pm 0.07$
<i>Heavy-chain shuffled library, selected on soluble antigen</i>			
C6H1	$5.9 \times 10^{-9}$	$11.0 \pm 0.50$	$6.2 \pm 0.12$
C6H2	$3.1 \times 10^{-9}$	$8.4 \pm 0.15$	$2.6 \pm 0.07$
<i>Combined scFv</i>			
C6H1L1	$1.5 \times 10^{-8}$	$4.1 \pm 0.18$	$6.2 \pm 0.11$
C6H2L1	$6.0 \times 10^{-9}$	$3.0 \pm 0.04$	$1.8 \pm 0.01$

$K_d$ ,  $k_{on}$  and  $k_{off}$  were determined by surface plasmon resonance in a BIAcore. Combined scFv result from combining the  $V_L$  of C6L1 with the  $V_H$  of either C6H1 or C6H2. ND, not determined.

must have been expressed as a pIII fusion due to read-through, albeit at low levels (Rogers *et al.*, 1992). The two scFv were subcloned and purified by IMAC and gel filtration. Both scFv were monomeric as determined by gel filtration (Figure 2). C6H1 had threefold higher affinity for c-erbB-2 than C6.5 and C6H2 had fivefold higher affinity than C6.5 (Table 4). The increased affinity of C6H1 ( $5.9 \times 10^{-9}$  M) was due to a faster  $k_{on}$ , whereas the increased affinity of C6H2 ( $3.1 \times 10^{-9}$  M) was due to both a faster  $k_{on}$  and a slower  $k_{off}$  (Table 4).

The expression level of C6H1 (opal stop codon) was reduced 200-fold compared to C6.5 (10 mg of purified C6.5/L of *E. coli* culture compared to 50  $\mu$ g/l for C6H1). This is consistent with observed expression levels in *E. coli* for the *lacI* gene with and without an opal codon (Rogers *et al.*, 1992). Background suppression of opal codons presumably inserts the amino acid tryptophan (Hirsh & Gold, 1971) or selenocysteine (Zinoni *et al.*, 1987). Tryptophan is the wild-type amino acid at this position in C6.5.

#### Location of mutations in chain shuffled scFv

Mutations in chain shuffled scFv were modeled on the Fv fragment of the immunoglobulin KOL (Marquart *et al.*, 1980; and see Figure 3). KOL was selected as the model because it has a  $V_L$  gene derived from the same family as C6.5, and a  $V_H$  gene with the same length CDR2. Mutations in higher-affinity scFv were located both in CDR residues at the antigen combining site, as well as at residues located far from the binding site (Tables 3 and 5, and Figure 3). All four light-chain shuffled scFv that formed mixtures of monomer and dimer had

mutations in residues that form the  $\beta$ -sheet that packs on the  $V_H$  domain (Table 3 and Figure 3). In contrast, scFv that did not form dimers (C6L1, C6H1, and C6H2) did not have mutations located in the  $V_H$ - $V_L$  interface, except for two conservative mutations located in  $V_H$  FR3 of C6H1 and C6H2 (V89M and F91Y; Tables 3 and 5, and Figure 3).

#### Affinities of scFv resulting from combining higher-affinity $V_H$ and $V_L$ genes obtained by chain shuffling

In an attempt to further increase affinity, shuffled rearranged  $V_H$  and  $V_L$  genes from higher-affinity scFv were combined into the same scFv. Combining the rearranged  $V_L$  gene from C6L1 with the rearranged  $V_H$  gene from C6H1 resulted in an scFv (C6H1L1) with lower affinity than either C6L1 or C6H1 (Table 4). Similarly, combining the rearranged  $V_L$  gene from C6L1 with the rearranged  $V_H$  gene from C6H2 resulted in an scFv (C6H2L1) with lower affinity than C6L1 or C6H2 (Table 4). Thus in both instances combining the independently isolated higher-affinity rearranged  $V_H$  and  $V_L$  genes did not have an additive effect on affinity.

#### Discussion

High-affinity scFv that bind the tumor antigen c-erbB-2 were engineered by shuffling the  $V_H$  gene segment and the rearranged  $V_L$  gene of an scFv isolated from a non-immune phage antibody library. The scFv were produced without any immunization, are entirely human in sequence, and the affinities ( $2.5 \times 10^{-9}$  M and  $3.1 \times 10^{-9}$  M) compare favorably with the affinities of hybridoma antibodies



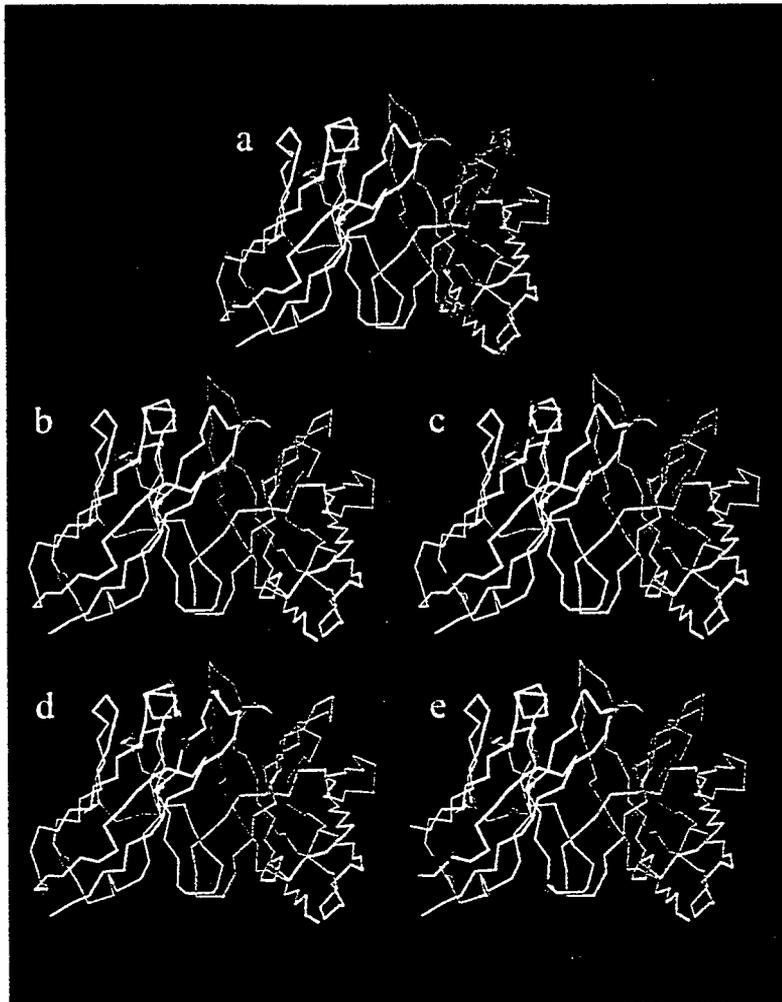


Figure 3. Location of mutations in light-chain and heavy-chain shuffled scFv. Amino acid residues that differ from the residues in C6.5 scFv are indicated as red spheres on the C $\alpha$  tracing of the Fv fragment of the immunoglobulin KOL (Marquart *et al.*, 1980). The V<sub>H</sub> domain is shown in green and the V<sub>L</sub> domain in yellow. a, Mutations in C6L1 are all located in the V<sub>L</sub> domain with parental V<sub>H</sub> sequence, mutations in C6H2 are all located in the V<sub>H</sub> domain, with parental V<sub>L</sub> sequence; b, C6VLB; c, C6VLD; d, C6VLE; e, C6VLF. scFv that form mixtures of monomer and dimer (C6VLB, C6VLD, C6VLE, and C6VLF; b to e) all have mutations located in the V<sub>H</sub>-V<sub>L</sub> interface. In contrast scFv that do not form dimers (C6L1, C6H1 (not shown), and C6H2, in a) do not have mutations located in the V<sub>H</sub>-V<sub>L</sub> interface, except for two conservative mutations located in V<sub>H</sub> FR3 of C6H2.

produced from mice immunized with the same antigen (Carter *et al.*, 1992; Adams *et al.*, 1993). Two of the scFv had a reduced  $k_{off}$ , which translated into greater retention on the surface of cells expressing *c-erbB-2*. The greater cell surface retention should translate into more specific *in vivo* tumor targeting. The scFv express well in *E. coli* as secreted native protein and can be purified in high yield in two steps, facilitating further *in vitro* and *in vivo* study.

Isolation of higher-affinity scFv was dependent on the selection conditions used. When selections were performed on antigen immobilized on polystyrene, scFv were isolated that existed in solution as mixtures of monomer and dimer. Dimerization and oligomerization have been observed with other scFv (Weidner *et al.*, 1992; Griffiths *et al.*, 1993; Marks *et al.*, 1993; Holliger *et al.*, 1993; Hughes-Jones *et al.*, 1994; Kortt *et al.*, 1994; Nissim *et al.*, 1994; Whitlow *et al.*, 1994; Deng *et al.*, 1995), and result from the V<sub>H</sub> domain of one scFv molecule pairing with the V<sub>L</sub> domain of a second scFv molecule, and *vice versa* (Holliger *et al.*, 1993; Whitlow *et al.*, 1994). The resulting homodimeric scFv have two binding sites, which can result in a significant increase in apparent affinity (avidity) when binding to multivalent antigen (Griffiths *et al.*, 1993; Holliger *et al.*, 1993; Kortt *et al.*, 1994; Whitlow *et al.*, 1994; Deng *et al.*, 1995). The tendency of scFv

to dimerize is sequence dependent, with some scFv existing as stable monomer (Griffiths *et al.*, 1993; Holliger *et al.*, 1993; Hughes-Jones *et al.*, 1994; Schier *et al.*, 1995), and others as mixtures of monomeric and oligomeric scFv (Griffiths *et al.*, 1993; Hughes-Jones *et al.*, 1994; Nissim *et al.*, 1994; Whitlow *et al.*, 1994; Deng *et al.*, 1995). Thus, a phage antibody library will consist of some phage with monomeric scFv on the surface, and other phage with dimeric scFv on the surface. Dimeric scFv can form on the phage surface by non-covalent association of the V domains of the scFv-pIII fusion with the V domains of native scFv in the periplasm. Native scFv appears in the periplasm by both incomplete suppression of the amber codon between the scFv gene and gene III, and by proteolysis. Our results demonstrate that dimeric scFv will be selected preferentially over monomeric scFv when selections are performed on immobilized antigen, due to avidity. This selection bias interferes with the selection of scFv with truly higher monovalent affinity and may explain the failure of Deng *et al.* (1995) to isolate higher-affinity anti-carbohydrate scFv from a phage display library selected on multivalent antigen immobilized on polystyrene. Instead, scFv with a greater tendency to dimerize were isolated. Our results also indicate that a relatively small number of amino acid substitutions (seven or less) can convert a mono-

meric scFv to an scFv, forming mixtures of monomer and dimer.

Experimental results suggest that scFv dimerization depends on the tendency of  $V_H$  and  $V_L$  domains to dissociate (Whitlow *et al.*, 1994). As measured on Fv fragments, the  $V_H$ - $V_L$  dissociation constant is typically high ( $10^{-6}$  M), but can differ at least 100-fold between different Fv ( $10^{-6}$  M to  $10^{-3}$  M; Horne *et al.*, 1982; Glockshuber *et al.*, 1990; Rodrigues *et al.*, 1995). When the dissociation constant is high, the  $V_H$  and  $V_L$  domains on the same scFv dissociate and pair with domains on another scFv molecule. Differences in the  $V_H$ - $V_L$   $K_d$  result from differences in residues composing the  $\beta$ -sheets that make up the  $V_H$ - $V_L$  interface (Chothia *et al.*, 1985). While many of these interface residues are conserved, 25% of the interface results from residues in the hypervariable CDRs (Chothia *et al.*, 1985). Interestingly, three of the four light-chain shuffled mutants that dimerize have substitutions in amino acid residues that comprise at least one of the  $\beta$ -strands in the interface. The fourth has an insertion in one of the interface  $\beta$ -strands. In three of these scFv, the mutations occur in CDR3. The effect of these mutations may be to reduce  $V_H$ - $V_L$  affinity, resulting in dissociation and subsequent dimer formation.

Isolation of higher-affinity monomeric scFv resulted from selections performed in solution on biotinylated antigen with subsequent capture on streptavidin magnetic beads. Selecting in solution reduces the avidity effect of dimeric scFv. For the initial rounds of selection, an antigen concentration greater than the  $K_d$  of the wild-type scFv was used in order to capture rare, or poorly expressed, phage antibodies. To select on the basis of affinity, an antigen concentration significantly less than the desired  $K_d$ , and less than the phage concentration, was used in the latter rounds of selection. In the case of  $V_L$  shuffling, higher-affinity binders were obtained with either of the antigen concentration regimens used, but the greatest enrichment for higher-affinity binders was obtained at the lowest antigen concentration ( $1.0 \times 10^{-11}$  M). In the case of  $V_H$  shuffling, higher-affinity binders were only obtained at the lowest antigen concentration ( $1.0 \times 10^{-11}$  M). Thus the greatest enrichment for higher-affinity binders was obtained by limiting the antigen concentration to less than the phage concentration (typically  $10^{-8}$  M) and the desired  $K_d$ . Alternatively, non-limiting antigen concentration has been used to select threefold higher affinity lysozyme binding scFv from a phage antibody library. In this case, however, a phage vector was used and 13 rounds of selection were utilized (Hawkins *et al.*, 1993), suggesting that selections using non-limiting antigen concentration are not as stringent. It is not possible to use 13 rounds of selection with a phagemid vector, since mutants with deleted antibody genes accumulate and take over the library (J. D. Marks, unpublished data). We prefer the use of a phagemid vector, due to its higher transformation efficiency and ability to easily produce native scFv.

Relative apparent enrichment ratios of phage antibodies are not only dependent on affinity, but are also affected by factors such as scFv expression level, folding efficiency, and level of toxicity to *E. coli*. Thus, the affinity of selected scFv will vary considerably (Riechmann & Weill, 1993), and a technique is needed to identify which of the selected clones are of higher affinity. A solid-phase based assay (inhibition ELISA; Friguier *et al.*, 1985) failed to identify higher affinity scFv when used to screen bacterial periplasms containing different concentrations of monomeric, dimeric, and aggregated scFv. This is consistent with differences observed in binding constants for Fab *versus* IgG determined by inhibition ELISA (Stevens, 1987). Therefore clones were ranked by measuring the  $k_{off}$  of scFv in bacterial periplasm using a BIAcore. Using the BIAcore, we could identify scFv with a slower  $k_{off}$  than the parental scFv without purification. Since a reduction in  $k_{off}$  is typically the major kinetic mechanism resulting in higher affinity when V genes are mutated, both *in vivo* (Foote & Milstein, 1991) and *in vitro*, (Marks *et al.*, 1992), this approach should generally result in the identification of higher-affinity scFv. Using this approach, we did not sequence, or subclone for purification, any scFv that did not have a higher affinity. In the case of heavy-chain shuffling, where only one in eight clones was of higher affinity, considerable effort was saved.

*In vivo*, low-affinity antibodies produced during the primary immune response utilize very few of the possible germline gene segments and have few point mutations in the V genes. Higher-affinity antibodies produced during the secondary and tertiary immune response utilize  $V_H$  and  $V_L$  gene segment pairings not observed during the primary immune response (repertoire shift) and accumulate point mutations in the rearranged V genes (Berek & Milstein, 1987; Foote & Milstein, 1991). Chain shuffling is the only *in vitro* mutagenesis technique that creates both repertoire shifted mutants and point mutation mutants. In the present example, all of the  $V_H$  and  $V_L$  gene segments of the higher-affinity scFv were derived from the same germline gene segments as the parental scFv. This was also the case when the  $V_H$  gene segment and rearranged  $V_L$  gene of a hapten binding scFv were shuffled (Marks *et al.*, 1992). This does not necessarily indicate that V genes derived from different germline genes did not produce a binding scFv, but rather that a higher-affinity scFv was not produced. While "promiscuous"  $V_H$  and  $V_L$  pairings occur (Clackson *et al.*, 1991; Collet *et al.*, 1992; Barbas *et al.*, 1993), even between chains from different species (Figini *et al.*, 1994), the data would suggest that these pairings are less likely to produce higher-affinity scFv.

The five- to sixfold increases in affinity achieved by heavy and light-chain shuffling are comparable to results achieved on protein binding antibody fragments using other mutagenesis techniques and phage display. For example, the affinity of an

anti-gp120 Fab was increased eightfold by sequential site-directed mutagenesis of  $V_H$  CDR1 and  $V_H$  CDR3 (Barbas *et al.*, 1994), and the affinity of an anti-lysozyme scFv was increased fivefold by error prone PCR mutagenesis (Hawkins *et al.*, 1993). Prior shuffling experiments of protein binding Fabs from human immune libraries resulted in Fabs of "similar apparent binding constants" (Collet *et al.*, 1992; Barbas *et al.*, 1993). The authors, however, appeared to be examining the "promiscuity" of  $V_H$  and  $V_L$  gene pairings, rather than attempting to use the technique for affinity maturation. Thus a relatively insensitive technique was used to measure affinities (competition ELISA). In addition, selections were not performed using antigen in solution. The five and sixfold increases in affinity achieved by chain shuffling the protein binding C6.5 are significantly less than the 20- and 15-fold increases in affinity achieved when shuffling an scFv that bound the hapten 2-phenyloxazol-5-one (Marks *et al.*, 1992). This difference may be due to the greater number of contact residues between an antibody and protein antigen, compared to a hapten; shuffling a protein binding antibody fragment would be more likely to result in disruption of favorable contacts, effectively reducing the library size. Alternatively, this difference could reflect the frequency of mutant chains in the library derived from the same germline gene as the parental scFv, and the extent of their diversification by somatic mutation. The rearranged  $V_L$  gene of C6.5 must occur rarely in the repertoire, since none of 92 unselected  $V_L$  shuffled scFv bound antigen. In contrast, 7/92 unselected  $V_L$  shuffled pHox binding scFv bound to antigen (J. D. Marks, unpublished data). The  $V_H$  gene segment of C6.5 is also likely to occur infrequently in the repertoire, since it is derived from a  $V_{H5}$  germline gene, a family frequently expressed in the fetal, but not adult, repertoire. To partially overcome this limitation, a  $V_{H5}$  gene segment-enriched library was created (20/92 unselected scFv binding antigen); however there is little diversity in the location of mutations (Table 5).

The  $k_{off}$  of the highest-affinity shuffled scFv ( $2.0 \times 10^{-3} s^{-1}$ ) translates into a theoretical  $t_{1/2}$  on the cell surface of less than ten minutes. This value correlates well with measured cell surface retention and may explain why so little scFv is retained in tumors *in vivo* at 24 hours (Adams *et al.*, 1993; Schier *et al.*, 1995). To achieve significant tumor retention at 24 hours, reduction of the  $k_{off}$  to  $<10^{-5} s^{-1}$  ( $t_{1/2}$  18 hours) is likely to be required, a value unlikely to be achieved with antibodies produced from hybridomas (Foote & Eisen, 1995). One approach in order to increase affinity further is to combine mutations that independently increase affinity (Wells, 1990; Hawkins *et al.*, 1993). Combining the  $V_H$  and  $V_L$  shuffled mutations, however, did not result in a further increase in affinity. The reason for the lack of additivity is unclear, but suggests that a sequential approach to chain shuffling (Marks *et al.*, 1992) may be more prudent. Nevertheless, it should

prove possible to further reduce the  $k_{off}$  of C6.5 by additional mutagenesis and selection. Availability of scFv mutants binding to the same c-erbB-2 epitope with a wide range of affinities would permit determination of the role of affinity in tumor targeting.

## Materials and Methods

### Construction of heavy-chain shuffled libraries

To facilitate heavy-chain shuffling, libraries were constructed in pHEN-1 (Hoogenboom *et al.*, 1991) containing human  $V_H$  gene segment repertoires (FR1 to FR3) and a cloning site at the end of  $V_H$  FR3 for inserting the  $V_H$  CDR3,  $V_H$  FR4, linker DNA and light chain from a binding scFv as a *Bss*HII-*Not*I fragment. To create the libraries, three  $V_H$  gene segment repertoires enriched for human  $V_{H1}$ ,  $V_{H3}$ , and  $V_{H5}$  gene segments were amplified by PCR using as a template single-stranded DNA prepared from a  $1.8 \times 10^8$  member scFv phage antibody library in pHEN-1 (Marks *et al.*, 1991). For PCR, 50  $\mu$ l reactions were prepared containing 10 ng template, 25 pmol back primer (LMB3), 25 pmol forward primer (PVH1FOR1, PVH3FOR1, or PVH5FOR1), 250  $\mu$ M dNTPs, 1.5 mM  $MgCl_2$ , and 0.5  $\mu$ l (2 units) *Taq* DNA polymerase (Promega) in the manufacturer's buffer. Primers PVH1FOR1, PVH3FOR1, and PVH5FOR1 were designed to anneal to the consensus  $V_{H1}$ ,  $V_{H3}$ , or  $V_{H5}$  3' FR3 sequence, respectively (Tomlinson *et al.*, 1992). The reaction mixture was subjected to 25 cycles of amplification (94°C for 30 s, 55°C for 30 s and 72°C for 30 s) using a Hybaid OmniGene cycler. The products were gel purified, isolated from the gel using DEAE membranes, eluted from the membranes with high salt buffer, precipitated with ethanol, and resuspended in 20  $\mu$ l of water (Sambrook *et al.*, 1990).

The DNA fragments from the first PCR were used as templates for a second PCR to introduce a *Bss*HII site at the 3'-end of FR3 followed by a *Not*I site. The *Bss*HII site corresponds to amino acid residues 93 and 94 (Kabat numbering; Kabat *et al.* (1987), see Table 5), and does not change the amino acid sequence (alanine-arginine). PCR was performed as described above using 200 ng purified first PCR product as template, the forward primers PVH1FOR2, PVH3FOR2, and PVH5FOR2, and the back primer LMB3. The PCR products were purified by extraction with phenol/chloroform (1:1, v/v), precipitated with ethanol, resuspended in 50  $\mu$ l water and 5  $\mu$ g digested with *Not*I and *Nco*I. The digested fragments were gel purified and each  $V_H$  gene segment repertoire was ligated separately into pHEN-1 (Hoogenboom *et al.*, 1991) digested with *Not*I and *Nco*I. The ligation mix was purified by extraction with phenol/chloroform (1:1, v/v), precipitated with ethanol, resuspended in 20  $\mu$ l water, and 2.5  $\mu$ l samples electroporated (Dower *et al.*, 1988) into 50  $\mu$ l *E. coli* TG1 (Gibson, 1984). Cells were grown in 1 ml SOC (Sambrook *et al.*, 1990) for 30 minutes and then plated on TYE medium (Miller, 1972) containing 100  $\mu$ g ampicillin/ml and 1% (w/v) glucose (TYE-AMP-Glu). Colonies were scraped off the plates into 5 ml of 2  $\times$  TY broth (Miller, 1972) containing 100  $\mu$ g ampicillin/ml, 1% glucose (2  $\times$  TY-AMP-Glu) and 15% (v/v) glycerol for storage at -70°C. The cloning efficiency and diversity of the libraries were determined by PCR screening (Gussov & Clackson, 1989) exactly as described by Marks *et al.* (1991). The resulting phage libraries were termed pHEN1- $V_{H1}$ rep, pHEN1- $V_{H3}$ rep, and pHEN1- $V_{H5}$ rep.

Three separate C6.5 heavy-chain shuffled phage antibody libraries were made from the pHEN1-V<sub>H</sub>1rep, pHEN1-V<sub>H</sub>3rep, and pHEN1-V<sub>H</sub>5rep phage libraries. The C6.5 light-chain gene, linker DNA, and V<sub>H</sub> CDR3 and FR4 were amplified by PCR from pHEN1-C6.5 (Schier *et al.*, 1995) plasmid DNA using the primers PC6VL1BACK and fdSEQ1. The PCR reaction mixture was digested with *Bss*HII and *Not*I and ligated into pHEN1-V<sub>H</sub>1rep, pHEN1-V<sub>H</sub>3rep, and pHEN1-V<sub>H</sub>5rep digested with *Not*I and *Bss*HII. Transformation and creation of library stocks was as described above.

### Construction of light-chain shuffled libraries

To facilitate light-chain shuffling, a library was constructed in pHEN1-V<sub>λ</sub>3 (Hoogenboom & Winter, 1992) containing rearranged human V<sub>κ</sub> and V<sub>λ</sub> gene repertoires, linker DNA, and cloning sites for inserting a rearranged V<sub>H</sub> gene as an *Nco*I-*Xho*I fragment. An *Xho*I site can be encoded at the end of FR4 without changing the amino acid sequence of residues 102 and 103 (serine-serine; Kabat *et al.*, 1987). To create the library, a rearranged V<sub>κ</sub> and V<sub>λ</sub> gene repertoire was amplified by PCR from a 1.8 × 10<sup>8</sup> member scFv phage antibody library in pHEN-1 (Marks *et al.*, 1991). PCR was performed as described above using 10 ng template, 25 pmol back primer (RJH1/2/6Xho, RJH3Xho, or RJH4/5Xho) and 25 pmol forward primer (fd-seq1). The back primers were designed to anneal to the first six nucleotides of the (G<sub>4</sub>S)<sub>3</sub> linker and either the J<sub>H</sub>1, 2, 6, J<sub>H</sub>3, or J<sub>H</sub> 4, 5 segments, respectively. The PCR reaction mixture was purified as described above, digested with *Xho*I and *Not*I, gel purified and ligated into pHEN1-V<sub>λ</sub>3 (Hoogenboom & Winter, 1992) digested with *Xho*I and *Not*I. Transformation of *E. coli* TG1, PCR screening, and creation of library stocks was as described above. The resulting phage library was termed pHEN1-V<sub>L</sub>rep.

The light-chain shuffled phage antibody library was made from pHEN1-V<sub>L</sub>rep. The rearranged C6.5 V<sub>H</sub> gene was amplified by PCR from pHEN1-C6.5 plasmid DNA (Schier *et al.*, 1995) using the primers PC6VH1FOR and LMB3. The PCR reaction mixture was purified, digested with *Xho*I and *Nco*I, gel purified and ligated into pHEN1-V<sub>L</sub>rep digested with *Xho*I and *Nco*I. Transformation of *E. coli* TG1, PCR screening, and creation of library stocks was as described above.

### Construction of scFv containing highest-affinity V<sub>H</sub> and V<sub>L</sub> genes obtained by chain shuffling

Two new scFv were made by combining the rearranged V<sub>L</sub> gene of the highest-affinity light-chain shuffled scFv (C6L1) with the rearranged V<sub>H</sub> gene of the highest-affinity heavy-chain shuffled scFv (C6H1 or C6H2). The C6L1 plasmid was digested with *Nco*I and *Xho*I to remove the C6.5 V<sub>H</sub> gene and gel purified. The rearranged V<sub>H</sub> gene of C6H1 or C6H2 was amplified by PCR using the primers LMB3 and PC6VH1FOR, digested with *Nco*I and *Xho*I and ligated into the previously digested C6L1 vector. Clones were screened for the presence of the correct insert by PCR fingerprinting and confirmed by DNA sequencing.

### Preparation of phage

To rescue phagemid particles from the libraries, 10 ml of 2 × TY-AMP-Glu were inoculated with an appropriate volume of bacteria (approximately 50 to 100 μl) from

the library stocks to give an A<sub>600</sub> of 0.3 to 0.5 and grown for 30 minutes, with shaking at 37°C. About 1 × 10<sup>12</sup> plaque-forming units of VCS-M13 (Stratagene) particles were added and the mixture was incubated at 37°C for 30 minutes without shaking, followed by incubation at 37°C for 30 minutes with shaking. Cells were spun down, resuspended in 50 ml 2 × TY broth containing 100 μg ampicillin/ml and 50 μg kanamycin/ml (2 × TY-AMP-KAN), and grown overnight, with shaking at 25°C. Phage particles were purified and concentrated by two PEG precipitations (Sambrook *et al.*, 1990), resuspended in 5 ml phosphate-buffered saline (PBS: 25 mM NaH<sub>2</sub>PO<sub>4</sub>, 125 mM NaCl, pH 7.0) and filtered through a 0.45 μm filter. The phage preparation consistently resulted in a titer of approximately 10<sup>13</sup> transducing units (t.u.)/ml ampicillin-resistant clones.

### Selection of phage antibody libraries

The light-chain shuffled library was selected using immunotubes (Nunc; Maxisorb) coated with 2 ml *c-erbB-2* extracellular domain (ECD; 25 μg/ml) in PBS overnight at room temperature (Marks *et al.*, 1991). The tube was blocked for one hour at 37°C with 2% skimmed milk powder in PBS (2% MPBS) and the selection, washing, and elution were performed exactly as described by Marks *et al.* (1991) using phage at a concentration of 5.0 × 10<sup>12</sup> t.u./ml. One third of the eluted phage was used to infect 10 ml log phase *E. coli* TG1, which were plated on TYE-AMP-Glu plates as described above. The rescue-selection-plating cycle was repeated three times, after which clones were analyzed for binding by ELISA.

All libraries were also selected using biotinylated *c-erbB-2* ECD and streptavidin-coated paramagnetic beads as described by Hawkins *et al.* (1992) but with some modifications. To prepare biotinylated antigen, 2.0 ml *c-erbB-2* ECD (1 mg/ml) was incubated with 5 mM NHS-LC-biotin (Pierce) overnight at 4°C and then purified on a Presto desalting column (Pierce). For each round of selection, 1 ml of phage (approximately 10<sup>13</sup> t.u.) was mixed with 1 ml PBS containing 4% skimmed milk powder, 0.05% Tween 20, and biotinylated *c-erbB-2* ECD. Affinity-driven selections were performed by decreasing the amount of biotinylated *c-erbB-2* ECD used for selection. Two selection schemes were used. In selection scheme 1 (S1) antigen concentrations of 100 nM, 50 nM, 10 nM, and 1 nM were used for selection rounds 1, 2, 3, and 4, respectively. In selection scheme 2 (S2) antigen concentrations of 40 nM, 1 nM, 100 pM, and 10 pM were used for selection rounds 1, 2, 3, and 4, respectively. The mixture of phage and antigen was gently rotated on an under-and-over-turntable for one hour at room temperature. To capture phage binding biotinylated antigen, streptavidin-coated M280 magnetic beads (Dynabeads, Dynal) were blocked with 2% MPBS for one hour at 37°C, and then added to the mixture of phage and antigen. In S1, 200 μl (round 1), 100 μl (round 2) or 50 μl (rounds 3 and 4) of beads were incubated with the phage-antigen mixture for 15 minutes, rotating on an under-and-over-turntable at room temperature. In S2, 100 μl (round 1) or 50 μl (rounds 2, 3, and 4) of beads were incubated with the phage-antigen mixture for 15 minutes (round 1), ten minutes (round 2), or five minutes (rounds 3 and 4). After capture of the phage, the Dynabeads were washed a total of ten times (three times in PBS containing 0.05% Tween 20 (TPBS), twice in TPBS containing 2% skimmed milk powder, twice in PBS, once in 2%MPBS, and twice in PBS) using a Dynal magnetic particle concentrator. The Dynabeads were resuspended in 1 ml PBS, and 300 μl

were used to infect 10 ml log phase *E. coli* TG1 which were plated on TYE-AMP-Glu plates.

### Initial scFv characterization

Initial analysis of chain shuffled scFv clones for binding to *c-erbB-2* was performed by ELISA using bacterial supernatant containing expressed scFv. Expression of scFv (De Bellis & Schwartz, 1990) was performed in 96-well microtitre plates exactly as described by Marks *et al.* (1991) with the following exception. After overnight growth and expression at 30°C, 50  $\mu$ l 0.5% Tween 20 was added to each well and the plates were incubated for four hours at 37°C, with shaking, to induce bacterial lysis and increase the concentration of scFv in the bacterial supernatant. For selection performed on Immunotubes, ELISA plates (Falcon 3912) were incubated with *c-erbB-2* ECD (2.5  $\mu$ g/ml) in PBS at 4°C overnight. For selections performed with biotinylated protein, Immunolon 4 plates (Dynatech) were incubated overnight at 4°C with Immunopure avidin (10  $\mu$ g/ml in PBS; Pierce). After washing three times with PBS to remove unbound avidin, wells were incubated with biotinylated *c-erbB-2* ECD as described by Schier *et al.* (1995). In both cases, binding of scFv to *c-erbB-2* ECD was detected with the mouse monoclonal antibody 9E10 (1  $\mu$ g/ml), which recognizes the C-terminal peptide tag (Munro & Pelham, 1986), and peroxidase-conjugated anti-mouse Fc antibody (Sigma), as described by Marks *et al.* (1991). Selected binders were further characterized by sequencing of the V<sub>H</sub> and V<sub>L</sub> genes (Sanger *et al.*, 1977). Putative germline gene segment derivation was determined by alignment to the VBASE sequence directory (Tomlinson *et al.*, 1995). The clone C6H1 was sequenced in both directions to confirm the presence of an opal (TGA) stop codon in CDR1. Sequence data have been deposited with GenBank; accession numbers U36535-U36559.

Screening of scFv for relative affinity was performed essentially as described by Friguet *et al.* (1985). Immunolon 4 ELISA plates (Dynatech) were coated with avidin in PBS (10  $\mu$ g/ml) at 4°C overnight. Biotinylated *c-erbB-2* ECD (5  $\mu$ g/ml) was added to the wells and incubated for 30 minutes at room temperature. Bacterial supernatant containing scFv was incubated with various concentrations of *c-erbB-2* (0 to 100 nM) at 4°C for one hour. The amount of free scFv was then determined by transferring 100  $\mu$ l of each mixture into the wells of the previously prepared ELISA plate and incubating for one hour at 4°C. Binding of scFv was detected as under ELISA screening and the IC<sub>50</sub> calculated as described by Friguet *et al.* (1985).

Screening of scFv by dissociation rate constant ( $k_{off}$ ) was performed using real-time biospecific interaction analysis based on surface plasmon resonance (SPR) in a BIAcore (Pharmacia Biosensor). Typically, 24 ELISA positive clones from each of the final two rounds of selection were screened. A 10 ml culture of *E. coli* TG1 containing the appropriate phagemid was grown and expression of scFv was induced with isopropyl- $\beta$ -d-thiogalactopyranoside (IPTG; De Bellis & Schwartz, 1990). Cultures were grown overnight at 25°C, scFv were harvested from the periplasm (Breitling *et al.*, 1991), and the periplasmic fraction was dialyzed for 24 hours against Hepes-buffered saline (10 mM Hepes, 150 mM NaCl, pH 7.4, HBS). In a BIAcore flowcell, approximately 1400 resonance units (RU) of *c-erbB-2* ECD (25  $\mu$ g/ml) in 10 mM acetate buffer (pH 4.5) were coupled to a CM5 sensor chip *via* the amine group on lysines using NHS-EDC chemistry (Johnsson *et al.*, 1991). Association and dissociation of undiluted

scFv in the periplasmic fraction was measured under a constant flow of 5  $\mu$ l/min. The apparent  $k_{off}$  was determined from the dissociation part of the sensorgram for each scFv analyzed (Karlsson *et al.*, 1991). Typically, 30 to 40 samples were measured during a single BIAcore run, with C6.5 periplasmic preparations analyzed as the first and final samples to ensure stability during the run. The flowcell was regenerated between samples using 2.6 M MgCl<sub>2</sub> in 10 mM glycine (pH 9.5) without significant change in the sensorgram baseline after analysis of more than 100 samples.

### Subcloning, expression and purification of scFv

To facilitate purification, shuffled scFv genes were subcloned (Schier *et al.*, 1995) into the expression vector pUC119 Sfi-NotmycHis, which results in the addition of a hexa-histidine tag at the C-terminal end of the scFv. Cultures (200 ml) of *E. coli* TG1 harboring one of the C6.5 mutant phagemids were grown, expression of scFv was induced with IPTG (De Bellis & Schwartz, 1990), and the culture was grown at 25°C overnight. scFv were harvested from the periplasm (Breitling *et al.*, 1991), dialyzed overnight at 4°C against 8 l of IMAC loading buffer (50 mM sodium phosphate, pH 7.5, 500 mM NaCl, 20 mM imidazole) and then filtered through a 0.2  $\mu$ m filter.

scFv were purified by IMAC (Hochuli *et al.*, 1988) exactly as described by Schier *et al.* (1995). To separate monomeric, dimeric and aggregated scFv, samples were concentrated to a volume <1 ml in a Centricon 10 (Amicon) and fractionated on a Superdex 75 column using a running buffer of HBS. The purity of the final preparation was evaluated by assaying a sample by SDS-PAGE. Protein bands were detected by Coomassie staining. The concentration was determined spectrophotometrically, assuming that an A<sub>280</sub> of 1.0 corresponds to an scFv concentration of 0.7 mg/ml.

### Measurement of affinity, kinetics, and cell surface retention

The  $K_d$  values of scFv were determined using surface plasmon resonance in a BIAcore. In a BIAcore flow cell, approximately 1400 resonance units (RU) of *c-erbB-2* ECD (90 kDa; McCartney, *et al.*, 1995; 25  $\mu$ g/ml in 10 mM sodium acetate, pH 4.5) were coupled to a CM5 sensor chip (Johnsson *et al.*, 1991). Association and dissociation rates were measured under continuous flow of 5  $\mu$ l/min using a concentration range from 50 to 800 nM.  $k_{on}$  was determined from a plot of  $(\ln(dR/dt))/t$  versus concentration, where  $R$  is response;  $t$ , is time (Karlsson *et al.*, 1991). To verify that differences in  $k_{on}$  were not due to differences in immunoreactivity, the relative concentrations of functional scFv was determined using SPR in a BIAcore (Karlsson *et al.*, 1993). Briefly, 4000 RU of *c-erbB-2* ECD were coupled to a CM-5 sensor chip and the rate of binding of C6.5 (RU/s) was determined under a constant flow of 30  $\mu$ l/s. Over the concentration range of  $1.0 \times 10^{-9}$  M to  $1.0 \times 10^{-7}$  M, the rate of binding was proportional to the log of the scFv concentration. Purified C6VLB, C6VLD, C6VLE, C6VLF, C6L1, C6H1, and C6H2 were diluted to the same concentration ( $1.0 \times 10^{-8}$  M and  $2.0 \times 10^{-8}$  M) as determined by A<sub>230</sub>. The rate of binding to *c-erbB-2* ECD was measured and used to calculate the concentration, based on the standard curve constructed from C6.5. Concentration determined by BIAcore was within 5% of the concentrations determined by A<sub>230</sub>.  $k_{off}$  was determined from the dissociation part of the sensorgram at the highest concentration of scFv analyzed

(Karlsson *et al.*, 1991). To exclude rebinding,  $k_{off}$  was determined for C6.5, C6L1, and C6H2 in the presence and absence of  $5.0 \times 10^{-7}$  M c-erbB-2 ECD. This was accomplished using the "kinject" command, resulting in the passage of either HBS or  $5.0 \times 10^{-7}$  M c-erbB-2 ECD in HBS over the CM5 chip at the beginning of the scFv dissociation.  $k_{off}$  was calculated during the first 45 seconds of dissociation, excluding the change in bulk refractive index due to the additional protein injection. No significant differences in  $k_{off}$  were observed. For example, the dissociation rate constant for C6L1 was  $1.8(\pm 0.14) \times 10^{-3} s^{-1}$  in the presence of c-erbB-2 ECD compared to  $2.0(\pm 0.07) \times 10^{-3} s^{-1}$  in HBS. Cell surface retention of C6.5 and C6L1 was determined exactly as described by Schier *et al.* (1995).

### Modeling of location of mutations

The location of mutations in shuffled scFv was modeled on the structure of the Fab KOL (Marquart *et al.*, 1980) using the program O (Jones *et al.*, 1991) on a Silicon Graphics workstation.

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Appendix 3

Schier R and Marks JD. Efficient in vitro affinity maturation of phage antibodies using BIAcore guided selections. Human Antibodies and Hybridomas, In press.

Efficient in vitro affinity maturation of phage antibodies using BIAcore guided selections

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Abbreviations: AMP, ampicillin; CDR, complementarity determining region; c-erbB-2 ECD, extracellular domain of c-erbB-2; cfu, colony forming units; ELISA, enzyme linked immunosorbent assay; GLU, glucose; HBS, hepes buffered saline, 10 mM hepes, 150 mM NaCl, pH 7.4; IPTG, isopropyl $\beta$ -D-thiogalactopyranoside;  $k_{on}$ , association rate constant;  $k_{off}$ , dissociation rate constant; MPBS, 2% skimmed milk powder in PBS; MTBS, 2% skimmed milk powder in TPBS; PBS, phosphate buffered saline, 25 mM  $\text{NaH}_2\text{PO}_4$ , 125 mM NaCl, pH 7.0; RU, resonance units; scFv, single-chain Fv fragment; SPR, surface plasmon resonance; TEA, triethylamine; TPBS, 0.05% v/v Tween 20 in PBS;  $V_H$ , immunoglobulin heavy chain variable region;  $V_L$ , immunoglobulin light chain variable region.

Keywords: phage display; antibody fragment; affinity maturation; surface plasmon resonance; BIAcore; c-erbB-2

## Abstract

Selection of higher affinity mutant phage antibodies has proven less than straightforward due to sequence dependent differences in phage antibody expression, toxicity to *E. coli*, and difficulty in eluting the highest affinity phage. These differences lead to selection for increased levels of expression or decreased toxicity rather than for higher affinity. In this work, we demonstrate how surface plasmon resonance as employed in the BIAcore can be used to increase the efficiency of phage antibody selections, yielding greater increments in affinity from a single library. A mutant phage antibody library was created by randomizing nine amino acids located in the V<sub>L</sub> CDR3 of C6.5, a human scFv which binds the tumor antigen c-erbB-2 with a K<sub>d</sub> of 1.6 × 10<sup>-8</sup> M. The library was subjected to five rounds of selection in solution using decreasing concentrations of biotinylated c-erbB-2. After each round of selection, polyclonal phage were prepared and the rate of binding to c-erbB-2 determined in a BIAcore under mass transport limited conditions. Determination of the rate of binding permitted calculation of the concentration, and hence percent, of binding phage present. Results were used to select the antigen concentration for the next round of selection. To determine the optimal eluent, polyclonal phage was injected in a BIAcore and eluted using one of five different solutions (10 mM HCl, 50 mM HCl, 100 mM HCl, 100 mM triethylamine, 2.6 M MgCl<sub>2</sub>). Differences were observed in eluent efficacy, which was reflected in significant differences in the affinities of phage antibodies isolated from the library after a round of selection using the different eluents. Use of the BIAcore to determine the optimal eluent and guide the antigen concentration used for selection yielded a C6.5 mutant with a 16 fold reduction in K<sub>d</sub> (K<sub>d</sub>=1.0 × 10<sup>-9</sup> M). This represents at least a twofold greater increment in affinity than previously obtained from a single library of phage antibodies which bind protein antigens.

## Introduction

Development of therapeutic antibodies has been limited by the immunogenicity of rodent antibodies, difficulties in adapting conventional hybridoma technology to produce human antibodies, and limits imposed on antibody affinity by the *in vivo* immune system<sup>1</sup>. The first two limitations have been largely overcome by the display of natural<sup>2</sup> and synthetic antibody variable region gene repertoires<sup>3</sup> on the surface of phage<sup>4,5</sup>. Human antibody fragments can be recovered from these libraries against virtually any antigen<sup>2,6-9</sup> with affinities for protein antigens ranging from  $10^{-6}$  M to  $10^{-8}$  M. Affinity of these primary isolates can be increased by creating mutant phage antibody libraries and selecting higher affinity antibodies<sup>10-14</sup>.

Efficient selection of higher affinity mutant phage antibodies has proven less than straightforward due to sequence dependent differences in phage antibody expression and in toxicity to *E. coli*. These differences can lead to selection for increased expression levels, or decreased toxicity, rather than for higher affinity. In the case of single-chain Fv (scFv) phage antibodies, selection is also complicated by the tendency of some scFv to dimerize. Dimeric scFv exhibit increased apparent affinity due to avidity and are preferentially enriched over monomeric scFv when selections are performed on antigen immobilized on a solid phase<sup>14</sup>. Thus selections must be carefully designed to ensure enrichment based on affinity, rather than expression level, toxicity to *E. coli*, or avidity. It has been previously shown that optimal selection of higher affinity scFv phage antibodies occurs when selections are performed in solution on biotinylated antigen with subsequent capture on streptavidin-coated magnetic beads<sup>10,14</sup>. For the initial round of selection, an antigen concentration greater than the  $K_d$  of the wild type scFv is used in order to capture rare, or poorly expressed, phage antibodies. In subsequent rounds, the antigen concentration is reduced to significantly less than the desired  $K_d$ <sup>14</sup>. Use of too high an antigen concentration results in failure to sort on the basis of affinity, while use of too low antigen concentration results in loss of binding

phage and subsequent overgrowth of deletion mutants <sup>14</sup>. The optimal antigen concentration cannot be predicted a priori, due to variability in phage antibody expression levels and uncertainty regarding the highest affinities present in the mutant phage antibody library. For selection of the highest affinity antibodies, it is also necessary to ensure that all specifically bound phage are eluted. Solutions used for elution include competition with soluble antigen <sup>10,15,16</sup>, 100 mM triethylamine (triethylamine (TEA)); <sup>2,6,7,10,11,16</sup>, glycine, pH 2.2 <sup>17-20</sup>, 100 mM NaOAc, pH 2.8 containing 500 mM NaCl <sup>21</sup>, or 76 mM citric acid, pH 2.8 <sup>22</sup>. Alternatively, magnetic beads with bound phage can be added directly to E. coli <sup>23</sup>.

For this work, we demonstrate how surface plasmon resonance (SPR) as employed in a BIAcore can be used to guide the antigen concentration used for selection during in vitro affinity maturation. Determination of the rate of binding of polyclonal phage to antigen after each round of selection permitted calculation of the concentration, and hence percent, of binding phage present. These values correlated closely with the number of positives observed by enzyme linked immunosorbent assay (ELISA). During successful phage antibody library selection, the values of bound phage remained high after each round, in the face of decreasing antigen concentration. Reduction of the antigen concentration below a critical level led to loss of binding of polyclonal phage to antigen in the BIAcore and a loss of ELISA positive clones. Using SPR, we also demonstrated that differences exist between eluents in their ability to elute polyclonal phage antibodies from antigen. These differences led to significant differences in the affinities of antibodies selected during in vitro affinity maturation. Selection of the highest affinity phage antibodies required use of the proper eluent, which could be predicted using SPR.

## Results

### Monitoring phage antibody selection using surface plasmon resonance in a BIAcore

A technique was developed to measure the concentration of antigen binding phage present in a polyclonal phage mixture. To construct a standard curve relating the concentration of binding phage to BIAcore response, phage were prepared from the anti-c-erbB-2 monoclonal phage antibody C6.5<sup>24</sup>. The phage concentration (colony forming units (cfu)/ml) was determined by titration on E. coli TG1. The response signal (resonance units; RU) and rate (RU/min) of binding of serial dilutions of C6.5 scFv phage to c-erbB-2 extracellular domain (c-erbB-2 ECD) were determined in a BIAcore under mass transport limited conditions<sup>25</sup>. A plot of the log of the phage concentration versus either the binding rate, or the amount of phage bound yielded linear standard curves (Figure 1).

To determine the utility of SPR for monitoring and guiding selections, we constructed a mutant phage antibody library C6VLCDR3 by randomizing nine amino acids located in the V<sub>L</sub> CDR3 of C6.5, a human scFv which binds the tumor antigen c-erbB-2 with a K<sub>d</sub> of  $1.6 \times 10^{-8}$  M<sup>24</sup>. After transformation, a library of  $1.0 \times 10^7$  clones was obtained. To isolate higher affinity scFv, the library was selected on decreasing concentrations of biotinylated c-erbB-2 ECD. After each round of selection, the concentration of binding phage were determined by SPR using the standard curves shown in Figure 1 (Table 1). The total phage concentration (cfu/ml) was determined by titration on E. coli TG1 and the percentage of antigen binding phage calculated as the [binding phage (BIAcore)]/[total phage (cfu/ml)]. The concentration of antigen used for the subsequent round of selection was reduced tenfold until the concentration of binding phage decreased significantly.

During the first four rounds of selection, the titre of eluted phage decreased as the antigen concentration used for selection decreased (Table 1). The concentration and percentage of binding phage as determined by SPR, however, increased each round

(Table 1). The percentage of individual colonies expressing scFv which bound c-erbB-2 ECD, as determined by ELISA, also increased each round, and the values correlated closely with the percent of binding phage determined by BIAcore (Table 1). These results suggest successful antigen driven selection. This was confirmed by measuring the  $K_d$  of native scFv expressed from 37 clones from the fourth round of selection (see Table 4 and the next section). All scFv had a lower  $K_d$  than the parental C6.5 scFv, with the best scFv having a 16 fold decreased  $K_d$ . Further reduction of the antigen concentration to  $1 \times 10^{-12}$  M in a fifth round of selection resulted in a large reduction of the BIAcore response, indicating loss of binding phage antibodies due to excessive stringency (Table 1). Loss of phage binding in the BIAcore correlated with an absence of binding as determined by ELISA. Loss of binding was paradoxically associated with an 800 fold increase in the titre of eluted phage (Table 1). PCR screening of 20 clones after the fifth round of selection indicated that most clones had lost part of the scFv gene.

#### BIAcore analysis and optimization of elution conditions for antibody phage selection

To determine if differences existed in the ability of eluents to remove antigen bound phage, polyclonal phage were prepared after three rounds of selection of the C6VLCDR3 library and studied using SPR in a BIAcore. After an initial bulk refractive index change, binding of phage to immobilized c-erbB-2 ECD was observed, resulting in an average of 189 RU bound (Table 2 and Figure 2). Phage were then allowed to either spontaneously dissociate from c-erbB-2 ECD using hepes buffered saline (HBS) as running buffer, or were eluted with either 100 mM HCl, 50 mM HCl, 10 mM HCl, 2.6 M  $MgCl_2$ , or 100 mM TEA. Major differences were observed between eluents in their ability to remove bound phage (Table 2 and Figure 2). The most effective solutions in removing bound phage antibodies were 100 mM HCl and 50 mM HCl, followed by 100 mM TEA. 2.6 M  $MgCl_2$  (which removes 100% of wild type C6.5) and 10 mM HCl were only minimally more effective than the running buffer in removing bound phage.

To determine if differences observed in the BIAcore were reflected in the affinity of selected scFv, a fourth round of selection was performed on the C6VLCDR3 phage antibody library. Phage were prepared from the third round of selection and elutions were performed using one of seven regimens: 1) 100 mM HCl; 2) 50 mM HCl; 3) 10 mM HCl; 4) 2.6 M MgCl<sub>2</sub>; 5) 100 mM TEA; 6) 1 μM c-erbB-2 ECD; and 7) no elution (magnetic beads resuspended in 1.5 ml 1 M Tris HCl pH 7.4). After the fourth round of selection, only minor differences were observed in the frequency of ELISA positive scFv (Table 3). The titre of eluted phage, however, was 6 to 30 times lower when elutions were performed with MgCl<sub>2</sub>, HCl, or TEA, compared to not eluting, or eluting with antigen (Table 3). To screen for the highest affinity scFv, native scFv was expressed from 24 ELISA positive clones and the dissociation rate constant ( $k_{off}$ ) determined without purification. scFv was purified from the eight clones with the lowest  $k_{off}$  from each of the seven elution regimens, the  $K_d$ ,  $k_{on}$ , and  $k_{off}$  determined, and the scFv gene sequenced (Table 4). scFv resulting from elutions with 50 mM HCl, 100 mM HCl, and 100 mM TEA had significantly lower  $K_d$  than scFv resulting from elutions with 10 mM HCl, 1 μM c-erbB-2 ECD, or no elution (Table 3, Table 4). Elution with 100 mM HCl resulted in selection of the two highest affinity clones (Table 4, C6ML3-9 and C6ML3-14), however the difference in average affinity between elution with 100 mM HCl, 50 mM HCl, or 100 mM TEA was not statistically significant (Table 3). The different eluents, however, did yield scFv with similar kinetic properties but different sequences in V<sub>L</sub> CDR3 (Table 4).

## Discussion

Phage display has proven to be a powerful tool for increasing antibody affinity<sup>10-14,16</sup>. To make the process efficient, however, it is essential to obtain the highest affinity clones from each mutant library. This is not necessarily straightforward, since enrichment ratios depend not only on affinity, but also on differences in expression level, folding efficiency, and toxicity to *E. coli*. Selection on the basis of affinity is optimal when selections are performed in solution and the antigen concentration is reduced each round<sup>14</sup>. Failure to adequately reduce the antigen concentration results in failure to sort on the basis of affinity, while too large a reduction results in loss of binding phage<sup>14</sup>. Moreover, the optimal antigen concentration cannot be predicted a priori, due to variability in phage antibody expression levels and uncertainty regarding the highest affinities present in the mutant phage antibody library. Our data indicate that the stringency of selections can be monitored in a BIAcore by measuring the concentration and percentage of binding phage present in polyclonal phage prepared after each round of selection. The results can then be used to determine the antigen concentration used for the next round of selection. As in our example, little or no change in the binding phage concentration indicates that the antigen concentration can be decreased significantly (at least tenfold) in the next round of selection. A rapid drop in binding phage between rounds suggests either the need to repeat the round using a higher antigen concentration, or a conservative change in the antigen concentration used for the subsequent round.

As an alternative to monitoring selections using BIAcore, the titre of eluted phage is frequently followed, a rising titre being indicative of positive selection<sup>10,18,23</sup>. In this and previous work<sup>14,26</sup>, however, we observed that positive selection on the basis of affinity occurred despite falling titres of eluted phage. When the titre of eluted phage did increase, there was a loss of binding phage, as determined by both BIAcore and ELISA. The mechanism for the increase in titre is unclear, however the majority of

these phage have deleted at least a portion of the scFv gene. This could result in increased infection efficiency, due to a greater number of wild type pIII on the phage surface, or reduced toxicity to E. coli from leaky scFv expression. Regardless, following the titre of eluted phage does not appear to be a useful monitor of selection during affinity maturation using limiting antigen concentrations.

One potential limitation of this technique is that the concentration of binding phage is also affected by the efficiency with which mutant scFv are expressed on phage relative to the expression level of the phage antibody used to construct the standard curve. This would be reflected in a greater difference between the number of binding phage determined by ELISA compared to the value determined by BIAcore. In selecting other C6.5 based libraries, we have observed differences as great as 1.5 fold between percentages of positive binders determined by BIAcore and ELISA. In these instances, the expression levels of native scFv was also significantly lower than the expression level of C6.5 (RS and JDM, unpublished data).

Our results also demonstrate the important effect of eluent choice on the affinities of selected antibodies, even when using limiting antigen concentration and BIAcore screening to identify the highest affinity scFv. Two previously described elution regimens were found to be the least effective for selecting higher affinity antibodies; infecting without elution by adding magnetic beads with antigen-bound phage directly to E. coli cultures,<sup>23</sup> and competitive elution of scFv with soluble antigen<sup>10,15,16</sup>. When eluting by incubating phage bound to antigen with E. coli, the phage probably must dissociate from antigen for infection to occur. Steric hindrance, due to the size of paramagnetic beads, blocks the attachment of pIII on antigen bound phage to the f-pilus on E. coli. This would result in preferential selection of scFv with rapid  $k_{off}$ , consistent with our results. Since a reduction in  $k_{off}$  is the major mechanism for decreases in  $K_d$ , this results in the selection of lower affinity scFv. Eluting with soluble antigen has a similar effect on the kinetics of selected scFv. The phage must first

dissociate from immobilized antigen, then rebinding is blocked by binding of the phage to soluble antigen. Phage antibodies with the lowest  $k_{\text{off}}$  will remain bound to immobilized antigen and therefore are not available for infection of E. coli.

The optimal type of eluent (acidic, basic, chaotropic) and concentration required will depend on the phage antibody affinity<sup>27,28</sup> and the type of bonds that need to be interrupted. This will vary considerably between libraries, depending on the nature of the antigen-antibody interaction. In this example, significantly higher affinity scFv were obtained eluting with HCl, pH 1.3 compared to HCl, pH 2.0. In fact, the affinities of scFv isolated after elution with HCl, pH 2.0 were no different than results obtained without eluting. Similarly, we studied 2.6 M MgCl<sub>2</sub>, because we had previously determined it would remove 100% of bound wild type C6.5<sup>24</sup>. This concentration of MgCl<sub>2</sub>, however, was ineffective in eluting C6.5 V<sub>L</sub> CDR3 mutants. Eluting with higher concentrations of MgCl<sub>2</sub> would have resulted in the selection of higher affinity scFv. For example, 3 M MgCl<sub>2</sub> was required to elute 100% of C6L1 scFv ( $K_d = 2.5 \times 10^{-9}$  M)<sup>14</sup> from a c-erbB-2 ECD BIAcore sensor chip and 4 M MgCl<sub>2</sub> was required to elute 100% of C6ML3-9 ( $K_d = 1.0 \times 10^{-9}$  M).

A convenient way to predict the optimal eluent is to analyze polyclonal phage in a BIAcore. The results can then be used to design elution conditions to achieve optimal enrichment for high affinity clones. One approach would be to elute sequentially, using a less stringent eluent to remove low affinity binders, followed by a more stringent eluent to remove high affinity binders. Thus the BIAcore information is used to select 'washing' reagents which remove low affinity phage antibodies more effectively than PBS. This could reduce the number of selection rounds and amount of screening required to select and identify the highest affinity binders. This strategy might also be useful to isolate antibodies to low density antigens on intact cells or tissue. A mild eluent could be used to remove low affinity phage antibodies, which are preferentially selected due to high density antigen present on the cell surface, as well as non-

specifically bound phage. Phage specific for lower density antigens would then be removed using a more stringent solution.

An alternative to eluting with stringent solutions is to use antigen biotinylated with NHS-SS-Biotin (Pierce) <sup>7</sup>. All of the bound phage can be released from the magnetic beads by reducing the disulfide bond between antigen and biotin. One advantage of this approach is that elution of all phage is guaranteed. Use of NHS-SS-Biotin could be combined with use of a milder eluent for washing (determined by BIAcore analysis) to increase enrichment for higher affinity phage antibodies. Our results suggest, however, that use of stringent eluents that are chemically different (acidic, basic, or chaotropic) results in the selection of scFv of equally high affinity, but of different sequence. Isolation of scFv of different sequences has a number of advantages. Single amino acid changes can affect expression levels in *E.coli* dramatically <sup>29</sup>. For example, expression level of C6ML3-5 (100 µg/L) was 100 times less than for wild type C6.5 (10 mg/L). Furthermore, different scFv might have different physicochemical characteristics (dimerization, stability, or immunoreactivity) or even different effects *in vivo* (specificity, biodistribution, or clearance). Thus parallel selections using different stringent eluents should result in a greater number of high affinity binders than use of a single eluent.

The  $K_d$  of C6.5 was decreased 16 fold from a single library of  $V_L$  CDR3 mutants. This is at least twofold greater than the three to eightfold decreases in  $K_d$  previously obtained from a single library for protein binding phage antibodies. We conclude that this greater efficiency in affinity maturation results from use of the BIAcore to monitor and guide selections, and use of the optimal eluent, rather than the specific CDR selected for mutagenesis <sup>12,13</sup>. For example, using BIAcore guidance, the  $K_d$  of C6ML3-9 was reduced an additional ninefold by randomizing four amino acids in  $V_H$  CDR3 (Schier et al., submitted for preparation). Use of the BIAcore to guide selections should decrease the number of libraries required to achieve the desired  $K_d$ .

## Materials and Methods

### Library construction and phage preparation

A mutant phage antibody library was constructed based on the sequence of C6.5, a human scFv isolated from a non-immune phage antibody library which binds the tumor antigen c-erbB-2 with a  $K_d = 1.6 \times 10^{-8} \text{ M}$ <sup>24</sup>. This library is described in detail in a separate publication (Schier et al., submitted for preparation). The mutant phage antibody library C6VLCDR3 was constructed by partially randomizing nine amino acids (residues 89-95b, Kabat numbering<sup>30</sup>) located in the  $V_L$  CDR3 of C6.5. The ratio of nucleotides was chosen so that the frequency of wild type amino acid was 49% at each position randomized. The mutant C6.5 scFv gene repertoire was digested with SfiI and NotI and ligated into the phagemid vector pCANTAB5E (Pharmacia) digested with SfiI and NotI. After transformation, a library of  $1.0 \times 10^7$  clones was obtained. For selection, phage were prepared as previously described<sup>14</sup>.

### Selection of the phage antibody library

The C6VLCDR3 library was subjected to five rounds of selection in solution on biotinylated c-erbB-2 ECD, as described in<sup>14</sup>, but with some modifications. After capture of phage, streptavidin-coated paramagnetic beads (Dynal) were washed a total of ten times (3 x phosphate buffered saline (25 mM  $\text{NaH}_2\text{PO}_4$ , 125 mM NaCl, pH 7.0, PBS) containing 0.05% Tween 20 (TPBS), 2 x TPBS containing 2% skimmed milk powder (MTPBS), 2 x PBS, 1 x PBS containing 2% skimmed milk powder (MPBS), and 2 x PBS) using a Dynal magnetic particle concentrator. The Dynabeads were resuspended in 1 ml PBS, and one third was used to infect 10 ml log phase *E. coli* TG1<sup>31</sup> which were plated on TYE plates containing 100  $\mu\text{g}/\text{ml}$  ampicillin and 1% glucose (TYE-AMP-GLU)<sup>32</sup>.

For determination of the effect of eluent, the fourth round of selection was repeated, exactly as described above, except that after washing, bound phage were eluted by adding 100  $\mu\text{l}$  of one of seven eluents: 1) 100 mM HCl pH 1.0; 2) 50 mM HCl

pH 1.3; 3) 10 mM HCl pH 2.0; 4) 2.6 M MgCl<sub>2</sub>; 5) 100 mM TEA; 6) 1 μM c-erbB-2 ECD; or 7) no elution (magnetic beads resuspended in 1 ml of PBS). After five minutes incubation with eluent (15 minutes for the incubation with 1 μM c-erbB-2 ECD), the supernatant was transferred to a new tube and the mixture neutralized by the addition of 1.5 ml of 1 M Tris HCl pH 7.4. 500 μl of the elution mixture was used to infect 10 ml log phase *E. coli* TG1<sup>31</sup> which were plated on TYE-AMP-GLU plates.

#### BIAcore and ELISA screening

Phage ELISA were performed to determine the percentage of antigen binding clones. 96 single colonies were picked from the unselected library and after each round of selection, resuspended in 200 μl 2xTY-AMP-GLU, and grown over night at 37°C in a 96-well microtitre plate (Corning). Aliquots of bacteria were transferred to a new 96-well microtitre plate containing 100 μl 2xTY-AMP-0.1% glucose and grown to an OD<sub>600</sub> of approximately 0.7. 50 μl of VCS-M13 helper phage (Stratagene) (2.5 × 10<sup>8</sup> pfu/) were added to each well, and the wells incubated for 1h at 37°C without shaking. 50 μl of 2 x TY-AMP containing 100 μg/ml kanamycin were added per well, and the bacteria grown overnight at 37°C. Bacteria were spun down at 2000 rpm in a Beckman GS-6R centrifuge and supernatant containing phage used for ELISA.

For phage ELISA, Immunolon 4 microtitre plates (Dynatech) were coated with 50 μl ImmunoPure avidin (Pierce; 10 μg/ml in PBS) overnight at 4°C, blocked with 1% bovine serum albumin in PBS for an hour at 37°C, incubated with 50 μl biotinylated c-erbB-2 ECD (5 μg/ml in PBS) for 30 minutes at 20°C, followed by an incubation with 50 μl *E. coli* supernatant containing phage for one hour at 20°C. Binding of scFv phage to the antigen was detected with a peroxidase-conjugated anti-M13 monoclonal antibody (Pharmacia) using ABTS as substrate. The reaction was stopped after 30 minutes with NaF (3.2 mg/ml) and the A<sub>405nm</sub> measured.

Screening of scFv by  $k_{off}$  was performed using real-time biospecific interaction analysis based on SPR in a BIAcore (Pharmacia) as described in<sup>14</sup>. Ten ml cultures of *E.*

E. coli TG1 containing the appropriate phagemid was grown and expression of scFv induced with isopropyl $\beta$ -D-thiogalactopyranoside (IPTG) <sup>33</sup>. Cultures were grown overnight at 25°C, scFv harvested from the periplasm <sup>34</sup>, and the periplasmic fraction dialyzed for 24 h against HBS. In a BIAcore flow cell, approximately 1400 RU of c-erbB-2 ECD (25  $\mu$ g/ml) in 10 mM acetate buffer pH 4.5 were coupled to a CM5 sensor chip using NHS/EDC amine coupling chemistry <sup>35</sup>. Dissociation of undiluted periplasmic fraction of E. coli containing scFv was measured under a constant flow of 5  $\mu$ l/min. An apparent  $k_{off}$  was determined from the dissociation part of the sensorgram for each scFv analyzed <sup>36</sup>.

#### Subcloning, expression and purification of scFv

To facilitate purification for kinetic studies, scFv genes were subcloned <sup>24</sup> into the expression vector pUC119 Sfi-NotmycHis, which results in the addition of a hexahistidine tag at the C-terminal end of the scFv. 500 ml cultures of E. coli TG1 harboring one of the C6.5 mutant phagemids were grown, expression of scFv induced with IPTG <sup>33</sup> and the culture grown at 25°C overnight. scFv was harvested from the periplasm <sup>34</sup>, and purified by immobilized metal affinity chromatography <sup>37</sup> exactly as previously described <sup>24</sup>. To remove dimeric and aggregated scFv, samples were concentrated to a volume < 1 ml in a Centricon 10 (Amicon) and fractionated on a Superdex 75 column using a running buffer of HBS. The purity of the final preparation was evaluated by assaying an aliquot by SDS-PAGE. The concentration was determined spectrophotometrically, assuming an  $A_{280}$  nm of 1.0 corresponds to an scFv concentration of 0.7 mg/ml.

#### Measurement of affinity and binding kinetics

The  $K_d$  of scFv were determined using SPR in a BIAcore. In a BIAcore flow cell, approximately 1400 RU of c-erbB-2 ECD (90 kDa; 25  $\mu$ g/ml in 10 mM sodium acetate, pH 4.5) were coupled to a CM5 sensor chip <sup>35</sup>. Association ( $k_{on}$ ) and  $k_{off}$  were measured under continuous flow of 5  $\mu$ l/min using a concentration range of scFv from 50 to 800

nM.  $k_{on}$  was determined from a plot of  $(\ln(dR/dt))/t$  vs concentration<sup>36</sup>. To verify that differences in  $k_{on}$  were not due to differences in immunoreactivity, the relative concentration of functional scFv was determined using SPR in a BIAcore as previously described<sup>14,25</sup>. The concentration of mutant scFv as determined by BIAcore was within 5% of the concentration determined by A<sub>280</sub>.  $k_{off}$  was determined from the first 1.5 minutes of the dissociation part of the sensorgram at the highest concentration of scFv analyzed<sup>36</sup>. To exclude rebinding,  $k_{off}$  was determined in the presence and absence of  $5.0 \times 10^{-7}$  M c-erbB-2 ECD as previously described<sup>14</sup>. No significant differences in  $k_{off}$  were observed between samples analyzed in the presence and absence of c-erbB-2 ECD.

#### Determination of efficacy of eluents in removing polyclonal phage from c-erbB-2 ECD

The efficacy of different elution solutions in removing polyclonal phage from c-erbB-2 ECD was determined using SPR in a BIAcore (Pharmacia). CM5 sensor chip flow cells were coated with 1800 RU of c-erbB-2 ECD (50  $\mu$ g/ml in 10 mM sodium acetate pH 4.5). Polyclonal phage were prepared<sup>14</sup> after the third round of selection and resuspended in HBS. 30  $\mu$ l of phage ( $5 \times 10^{12}$  cfu/ml) were injected over the flow cell surface using HBS as running buffer and a flow rate of 5  $\mu$ l/min. 2.5 minutes into the dissociation period, 5  $\mu$ l of one of six eluents (HBS, 2.6 M MgCl<sub>2</sub>, 100 mM TEA, 10 mM HCl, 50 mM HCl, or 100 mM HCl) was injected over the flow cell surface at a rate of 5  $\mu$ l/min, followed by a wash step. The amount of phage bound was determined 15 seconds after the end of the association phase and six minutes later at the end of the wash step. The differences between these two points was used to calculate the percent of phage still bound after elution.

#### Determination of the percent of binding phage in a polyclonal phage preparation

A standard curve was constructed using monoclonal C6.5 scFv phage<sup>25</sup>. Phage were prepared and titred (cfu/ml) on E. coli TG1<sup>14</sup>. In a BIAcore, 1800 RU of c-erbB-2 ECD was coupled to a CM5 sensor chip using NHS-EDC chemistry. Thirty ml aliquots of C6.5 phage ( $1.0 \times 10^{11}$  to  $1.0 \times 10^{13}$  cfu/ml) were injected over the flow cell surface

using a running buffer of PBS containing 0.05% P20 and a flow rate of 5  $\mu$ l/min. Binding rates were calculated in RU/min from the association portion of each sensorgram. The amount of phage bound (RU) was also determined using a reference point taken 15 sec after the end of the association phase. Two standard curves were constructed, one plotting the log of the phage titre vs the log of the binding rate, and one plotting the log of the phage titre vs the log of the RU phage bound (Figure 1).

To determine the concentration of binding phage in a polyclonal phage mixture, phage were prepared after each round of selection and the titre determined <sup>14</sup>. Thirty ml aliquots of phage ( $3.0$  to  $8.0 \times 10^{12}$  cfu/ml) were injected over the flow cell surface, the binding rate and RU bound measured, and the concentration (cfu/ml) of c-erbB-2 binding phage determined from the standard curves. The percent binding phage was calculated as the ratio of the concentration of binding phage (cfu/ml)/total phage titre determined by infection of E. coli (cfu/ml).

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## Figure Legends:

**Figure 1: Correlation between phage binding in a BIAcore and the phage titre.** Monoclonal C6.5 scFv phage ( $1.0 \times 10^{11}$  to  $1.0 \times 10^{13}$  cfu/ml) were injected over a c-erbB-2 ECD coupled sensor chip in a BIAcore and the rate of binding (RU/min) and amount (RU) of phage bound determined. Two standard curves were constructed, one plotting the log of the phage concentration (cfu/ml) vs the log of the binding rate (RU/min) (square symbol), and one plotting the log of the phage concentration (cfu/ml) vs the log of the amount of phage bound (RU) (round symbol). These standard curves were used to calculate the titre of binding phage in polyclonal mixtures of phage.

**Figure 2. Effect of different eluents on removing bound phage from c-erbB-2 ECD.** Polyclonal anti-c-erbB-2 phage were injected over a c-erbB-2 ECD coupled sensor chip in a BIAcore and the ability of six different eluents to remove bound phage was determined. A) overlay plot of the six sensorgrams generated from analysis of the six eluents: 1) baseline, beginning of association; 2) beginning of dissociation; difference between point 1 and 2 = amount of phage bound; 3) beginning of elution, differences in refractive index of eluents results in large positive or negative changes in RU, depending on the eluent used; 4) washing out of eluent from the flowcell; 5) amount of phage bound after elution. B) Enlargement of (A) between points 3 and 5. Significant differences exist in amount of phage remaining bound after elution. (I : HBS; II : 2.6 M  $MgCl_2$ ; III : 10 mM HCl; IV : 100 mM TEA; V : 50 mM HCl; VI : 100 mM HCl)

**Table 1. Results of selection of the C6VLCDR3 phage antibody library on c-erbB-2 ECD.** A phage antibody library consisting of V<sub>L</sub> CDR3 mutants of C6.5 scFv was subjected to five rounds of selection using decreasing antigen concentration (column 2). After each round of selection, the titre of eluted phage (column 3) was measured, the percent of individual clones binding antigen (column 4) was determined by ELISA, and polyclonal phage was prepared and titred for the next round of selection (column 5). The amount (RU) of polyclonal phage binding to a c-erbB-2 ECD coupled sensor chip was measured in a BIAcore (column 6) and used to determine a titre of binding phage (column 7) from a standard curve constructed using known concentrations of the monoclonal phage antibody C6.5 (see Figure 1). The percent of binding phage calculated by BIAcore (column 8 = (column 7/column 6)) correlated closely with the percentage of individual clones binding c-erbB-2 ECD by ELISA (column 4).

Round of selection	Antigen conc. used for selection [x 10 <sup>-9</sup> M]	Titre of eluted phage [phage / ml]	Phage binding by ELISA (%)	Titre of phage preparation [x 10 <sup>12</sup> phage/ml]	BIAcore response [RU]	BIAcore phage titre [x 10 <sup>12</sup> phage/ml]	BIAcore phage binding (%)
0	---	1.0 x 10 <sup>7</sup>	0	3.0	41	0.2	6
1	40	1.5 x 10 <sup>5</sup>	11	11.0	70	0.5	5
2	1	4.0 x 10 <sup>4</sup>	73	2.5	159	2.0	80
3	0.1	2.0 x 10 <sup>4</sup>	86	2.0	191	1.6	80
4	0.01	1.0 x 10 <sup>4</sup>	100	1.7	227	1.6	94
5	0.001	8.0 x 10 <sup>6</sup>	3	5.0	49	0.3	6

Table 2. Effects of different eluents on removing bound phage from c-erbB-2 ECD as determined by surface plasmon resonance in a BIAcore. Polyclonal anti-c-erbB-2 phage prepared after the third round of selection were injected over c-erbB-2 ECD coupled to a sensor chip in a BIAcore. After association, the amount (RU) of bound phage was determined, one of six eluents injected over the sensor chip surface, and the amount of phage that remained bound to c-erbB-2 determined. Major differences were observed in the efficacy of eluents in removing bound phage.

Eluent	RU phage bound before elution	RU phage bound after elution	% bound phage eluted
Hepes buffered saline	190	150	21
2.6 M MgCl <sub>2</sub>	192	141	27
100 mM triethylamine	195	84	57
10 mM HCl	189	127	33
50 mM HCl	182	0	100
100 mM HCl	185	0	100

**Table 3. Effect of different eluents on the selection of higher affinity phage antibodies.** Polyclonal phage were subjected to a fourth round of selection on c-erbB-2 ECD and the bound phage eluted with one of seven eluents (column 1). The titre of eluted phage (column 2) and number of individual clones binding c-erbB-2 was determined by ELISA. scFv with the lowest  $k_{off}$  were identified by BIAcore screening, the scFv purified, and the binding kinetics ( $k_{on}$  and  $k_{off}$ ) determined by BIAcore and used to calculate the  $K_d$ . Significant differences in the  $K_d$  of selected scFv were observed.

Eluent	Titre of eluted phage	ELISA positive clones	$K_d$ ( $\times 10^{-9}$ M)	$k_{on}$ ( $\times 10^5$ s $^{-1}$ M $^{-1}$ )	$k_{off}$ ( $\times 10^{-3}$ s $^{-1}$ )
No elution <sup>a</sup>	$5.2 \times 10^4$	75/92	$5.39 \pm 0.73$	$4.64 \pm 0.35$	$2.49 \pm 0.41$
1 $\mu$ M c-erbB-2	$6.0 \times 10^4$	82/92	$5.99 \pm 1.12$	$5.09 \pm 0.27$	$2.58 \pm .47$
2.6 M MgCl <sub>2</sub>	$1.1 \times 10^4$	83/92	$3.30 \pm 0.45^c$	$5.05 \pm 0.43$	$1.58 \pm .14$
100 mM TEA	$1.2 \times 10^4$	89/92	$2.65 \pm 0.35^b$	$4.78 \pm 0.39$	$1.27 \pm .20$
10 mM HCl	$1.0 \times 10^4$	85/92	$6.09 \pm 1.29$	$5.72 \pm 0.30$	$3.46 \pm .80$
50 mM HCl	$1.0 \times 10^4$	90/92	$2.60 \pm 0.40^b$	$6.38 \pm 1.02$	$1.54 \pm .19$
100 mM HCl	$2.1 \times 10^3$	87/92	$2.52 \pm 0.46^b$	$5.99 \pm 0.37$	$1.40 \pm .20$

a = magnetic beads with bound phage added directly to E. coli culture;

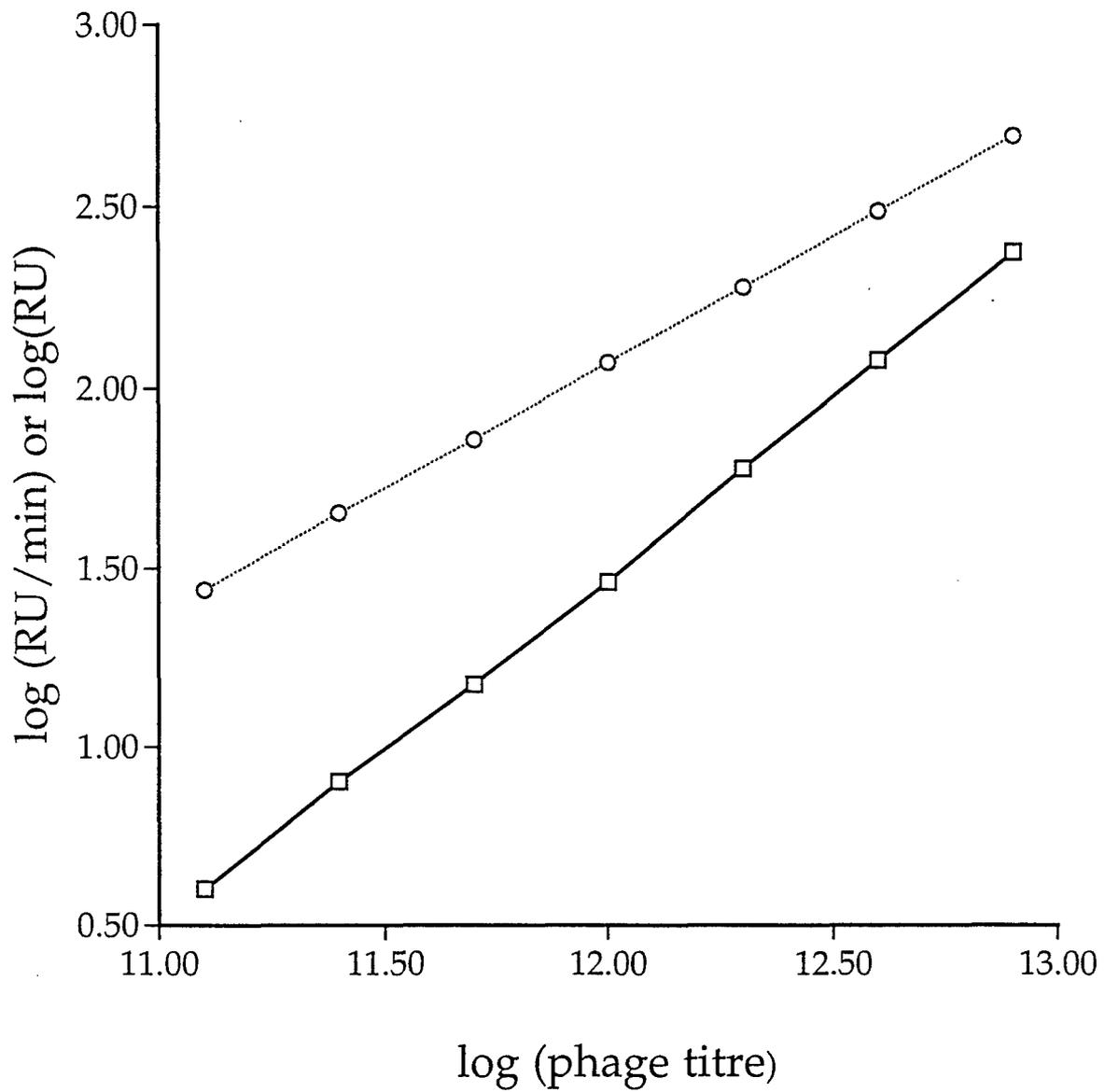
b =  $p < 0.05$  compared to no elution, 1  $\mu$ M c-erbB-2, and 10 mM HCl;

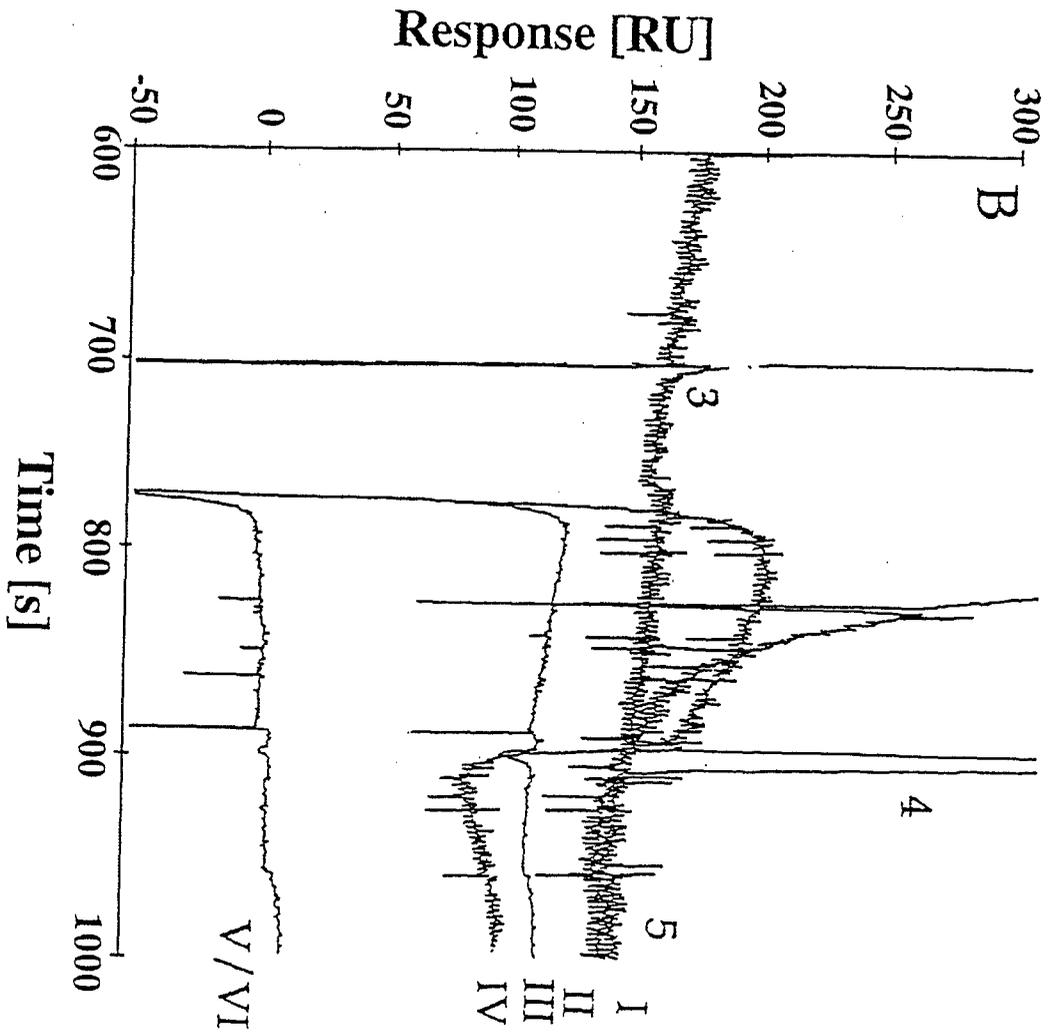
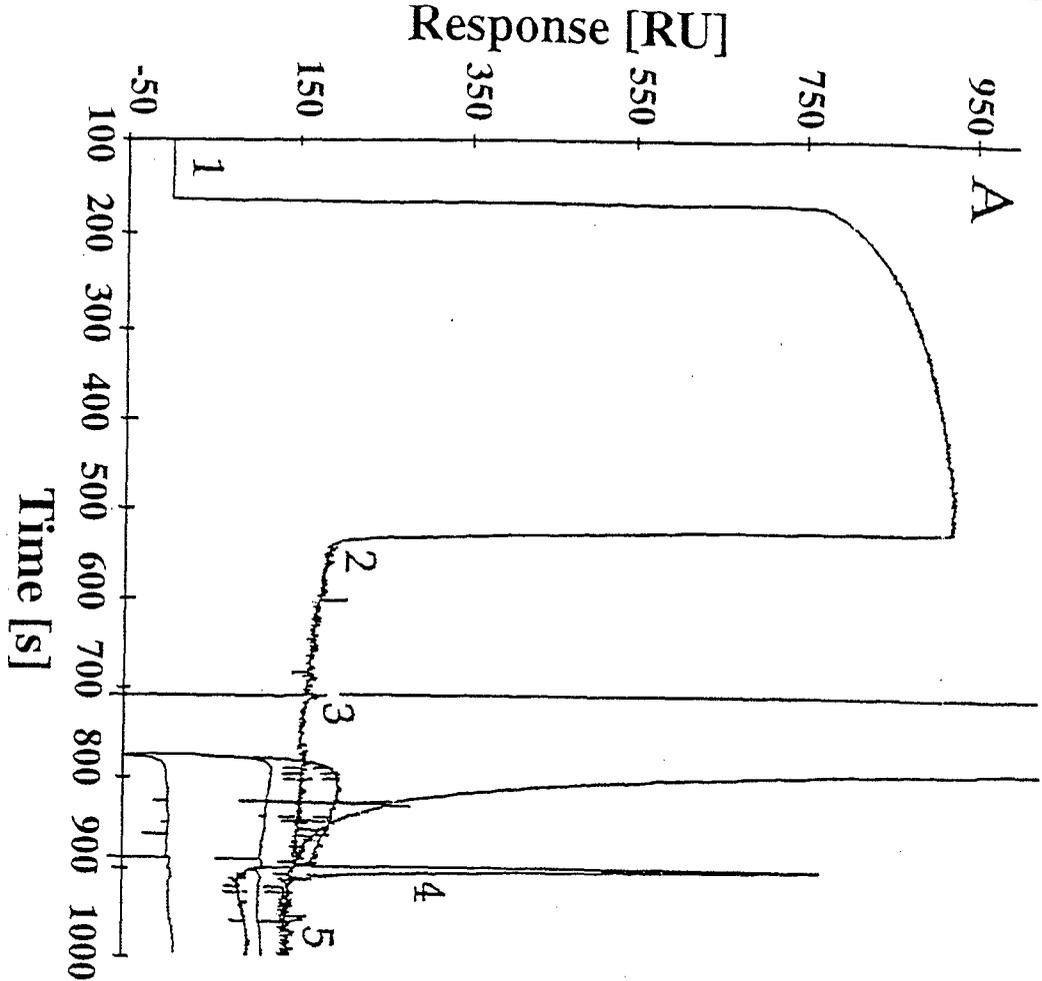
c =  $p < 0.05$  compared to 10 mM HCl.

**Table 4. Effect of elution solutions on the sequences, affinities and binding kinetics of purified scFv.**

Clone	V <sub>L</sub> CDR3 sequence	K <sub>d</sub> (x 10 <sup>-9</sup> M)	k <sub>on</sub> (x 10 <sup>5</sup> s <sup>-1</sup> M <sup>-1</sup> )	k <sub>off</sub> (x 10 <sup>-3</sup> s <sup>-1</sup> )
C6.5	AAWDDSLSGWV	16.0	4.0 ± 0.20	6.3 ± 0.06
<b>NO ELUTION</b>				
C6ML3-5 (4)	----Y-----	3.7	5.1 ± 0.34	1.9 ± 0.09
C6ML3-17	-S--YYR----	4.9	3.5 ± 0.22	1.7 ± 0.02
C6ML3-1	----Y--W----	6.1	3.3 ± 0.07	2.0 ± 0.15
C6ML3-22	----A-----	8.3	4.3 ± 0.21	3.6 ± 0.02
C6ML3-26	-----R----	8.3	6.0 ± 0.77	5.0 ± 0.04
<b>1 μM c-erbB-2</b>				
C6ML3-5 (5)	----Y-----	3.7	5.1 ± 0.34	1.9 ± 0.09
C6ML3-17	-S--YYR----	5.0	3.5 ± 0.22	1.7 ± 0.02
C6ML3-25 (2)	----NRH----	7.4	5.9 ± 0.72	4.4 ± 0.03
<b>2.6 M MgCl<sub>2</sub></b>				
C6ML3-12	----Y-R----	1.6	4.5 ± 0.16	0.72 ± 0.02
C6ML3-15	----RP-W----	2.2	5.9 ± 0.8	1.3 ± 0.02
C6ML3-7 (2)	----YAV----	2.6	6.5 ± 0.29	1.7 ± 0.09
C6ML3-5 (2)	----Y-----	3.7	5.1 ± 0.34	1.9 ± 0.09
C6ML3-16	-S--Y-R----	3.8	5.5 ± 0.12	2.1 ± 0.05
C6ML3-17	-S--YYR----	4.9	3.5 ± 0.22	1.7 ± 0.02
<b>100 mM TEA</b>				
C6ML3-19	-S--RP-W----	1.5	6.6 ± 0.69	1.0 ± 0.02
C6ML3-12 (2)	----Y-R----	1.6	4.5 ± 0.16	0.72 ± 0.02
C6ML3-18	-S--A--W----	2.4	2.6 ± 0.08	0.62 ± 0.02

C6ML3-20	---EQ--W---	3.0	4.7 ± 0.08	1.4 ± 0.02
C6ML3-5 (3)	----Y-----	3.7	5.1 ± 0.34	1.9 ± 0.09
<b>10 mM HCl</b>				
C6ML3-23	-S--H--W---	1.5	6.7 ± 0.41	1.0 ± 0.02
C6ML3-7	----YAV----	2.6	6.5 ± 0.29	1.7 ± 0.09
C6ML3-5	----Y-----	3.7	5.1 ± 0.34	1.9 ± 0.09
C6ML3-21	----Y-Q----	4.5	4.9 ± 0.01	2.2 ± 0.05
C6ML3-25	----NRH----	7.4	5.9 ± 0.72	4.4 ± 0.03
C6ML3-22	----A-----	8.3	4.3 ± 0.21	3.6 ± 0.02
C6ML3-26	-----R----	8.3	6.0 ± 0.77	5.0 ± 0.04
C6ML3-24	----EQIF----	12.4	6.4 ± 0.89	7.9 ± 0.04
<b>50 mM HCl</b>				
C6ML3-12 (2)	----Y-R----	1.6	4.5 ± 0.16	0.72 ± 0.02
C6ML3-29	----GT-W---	1.7	12.9 ± 1.03	2.2 ± 0.02
C6ML3-28	-S--YA-----	2.5	6.8 ± 0.17	1.7 ± 0.02
C6ML3-7 (2)	----YAV----	2.6	6.5 ± 0.29	1.7 ± 0.09
C6ML3-6	-S--Y-----	3.2	5.9 ± 0.43	1.9 ± 0.02
C6ML3-17	-S--YYR----	4.9	3.4 ± 0.22	1.7 ± 0.02
<b>100 mM HCl</b>				
C6ML3-9	-S--YT-----	1.0	7.6 ± 0.20	0.76 ± 0.03
C6ML3-14 (2)	-----P-W---	1.1	7.0 ± 0.40	0.77 ± 0.02
C6ML3-15	----RP-W---	2.2	5.9 ± 0.80	1.3 ± 0.02
C6ML3-5 (4)	----Y-----	3.7	5.1 ± 0.34	1.9 ± 0.09





Appendix 4

Schier R, Balint RF, McCall A, Apell G, Larrick JW, Marks JD. Identification of functional and structural amino acid residues by parsimonious mutagenesis. *Gene*, 169: 147-155, 1996.

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## Identification of functional and structural amino-acid residues by parsimonious mutagenesis

(c-erbB-2; single-chain Fv; affinity maturation; random mutagenesis; phage display)

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### SUMMARY

For in vitro evolution of protein function, we previously proposed using parsimonious mutagenesis (PM), a technique where mutagenic oligodeoxynucleotides (oligo) are designed to minimize coding sequence redundancy and limit the number of amino acid (aa) residues which do not retain parental structural features. For this work, PM was used to increase the affinity of C6.5, a human single-chain Fv (scFv) that binds the glycoprotein tumor antigen, c-erbB-2. A phage antibody library was created where 19 aa located in three of the heavy (H) and light (L) chain antigen-binding loops (L1, L3 and H2) were simultaneously mutated. After four rounds of selection, 50% of scFv had a lower dissociation rate constant ( $k_{off}$ ) than the parental scFv. The  $K_d$  of these scFv ranged from twofold ( $K_d = 7.0 \times 10^{-9}$  M) to sixfold ( $K_d = 2.4 \times 10^{-9}$  M) lower than the parental scFv ( $K_d = 1.6 \times 10^{-8}$  M). In higher affinity scFv, substitutions occurred at 10/19 of the positions, with 21/28 substitutions occurring at only four positions, two in H2, and one each in L1 and L3. Only the wild type (wt) aa was observed at 9/19 aa. Based on a model of C6.5, seven of the nine conserved aa have a structural role in the variable domain, either in maintaining the main chain conformation of the loop, or in packing on the H-chain variable domain. Two of the conserved aa are solvent exposed, suggesting they may play a critical role in recognition. Thus, PM identified three types of aa: structural aa, functional aa which modulate affinity, and functional aa, which are critical for recognition. Since the sequence space was not completely sampled, higher affinity scFv could be produced by subjecting functional aa which modulate affinity to a higher rate of mutation. Furthermore, PM could prove useful for modifying function in other proteins that belong to structurally related families.

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Abbreviations: aa, amino acid(s); Ab, antibody(ies); Ag, antigen(s); bp, base pair(s); CDR, complementarity determining region(s); c-erbB-2, glycoprotein tumor antigen; ECD, c-erbB-2 extracellular domain; ELISA, enzyme-linked immunosorbent assay; FR, framework region; H1, H2 and H3, first, second and third heavy-chain variable-

region antigen-binding loops; HBS, hepes-buffered saline (10 mM Hepes/150 mM NaCl pH 7.4); kb, kilobase(s) or 1000 bp;  $k_{on}$ , association rate constant;  $k_{off}$ , dissociation rate constant; L1, L2 and L3, first, second and third light-chain variable-region antigen-binding loops; nt, nucleotide(s); oligo, oligodeoxyribonucleotide; PCR, polymerase chain reaction; PM, parsimonious mutagenesis; scFv, single-chain Fv fragment; SPR, surface plasmon resonance;  $V_H$ , immunoglobulin heavy-chain variable region;  $V_L$ , immunoglobulin light-chain variable region;  $V_{\lambda}$ , immunoglobulin lambda light-chain variable region; wt, wild type.

## INTRODUCTION

Development of therapeutic antibodies (Ab) has been limited by the immunogenicity of rodent Ab, difficulties in adapting conventional hybridoma technology to produce human Ab and limits imposed on Ab affinity by the *in vivo* immune system (Foote and Eisen, 1995). The first two limitations have been largely overcome by the display of natural (Marks et al., 1991) and synthetic Ab variable region gene repertoires (Hoogenboom and Winter, 1992) on the surface of phage (McCafferty et al., 1990; Hoogenboom et al., 1991). Human Ab fragments can be recovered from these libraries against virtually any antigen (Ag), including haptens, foreign proteins, cell surface Ag and self-Ag (Marks et al., 1991; 1993; Griffiths et al., 1993; 1994). The affinities of Ab to protein Ag, however, range from  $10^{-6}$  to  $10^{-8}$  M, and need to be increased to achieve the affinities required for therapeutic use ( $<10^{-9}$  M).

Phage display can also be used to increase the affinity of Ab fragments isolated from phage Ab libraries (Hawkins et al., 1992; Marks et al., 1992; Barbas et al., 1994). The sequence of a binding phage Ab is diversified and higher-affinity binders selected from the mutant Ab library. Since it is difficult to make libraries larger than  $10^7$  to  $10^8$  clones, decisions must be made as to which aa to diversify, and to what extent. One approach is suggested by structural and functional analysis of the Ab combining site. Typically, 15–22 aa in the combining site of an Ab contact a similar number of aa in Ag (Davies et al., 1990). However free energy calculations and mutational analysis indicate that only a small subset of the contact residues contribute the majority of the binding energy (Novotny et al., 1989; Hawkins et al., 1993; Kelley and O'Connell, 1993). For the rest of the aa, a decrease in entropy accounts for most of the enthalpy decrease, resulting in no net effect on affinity (Novotny et al., 1989; Kelley and O'Connell, 1993). In many instances, 'repulsive contacts' are also made, which can cost up to several kcal (Novotny et al., 1989). Thus, Ab affinity could be increased by exchanging low affinity or repulsive contacts for higher-affinity contacts while retaining the few aa which contribute the majority of the binding energy. The problem is how to identify these aa, in the absence of high resolution structural and functional data.

Analysis of Ab combining sites indicates that the majority of the contact residues are located in six hyper-variable loops (reviewed in Wilson and Stanfield, 1993). The limits of the loops are defined structurally as lying outside of the  $\beta$ -sheet (Chothia and Lesk, 1987; Chothia et al., 1992). The length of human L1, L2, L3, H1 and H2 can vary from three to ten aa, with H3 lengths as long as 18 aa (Chothia and Lesk, 1987; Kabat et al., 1987;

Chothia et al., 1992). Thus up to 51 aa residues need to be scanned. Conventional oligo-directed mutagenesis uses the nucleotides (nt) NNS to randomize each aa. All parental contacts are discarded and the number of aa that can be scanned is limited to five, given typical transformation efficiencies. A greater number of aa can be scanned by PM, using oligos designed to minimize coding sequence redundancy and limit the number of aa which do not retain wt structural features (Balint and Larrick, 1993). Redundancy is reduced using (doping) codons where degeneracy is equal to or only slightly larger than the subsets of aa encoded. Non-viable structures are minimized by using biased (spiked) nt mixtures which bias for the wt aa and take advantage of the tendency of the genetic code to favor chemically or sterically conservative aa changes.

To determine the utility of PM, the technique was used to increase the affinity of a c-erbB-2 binding human scFv (C6.5) isolated from a non-immune phage Ab library (Schier et al., 1995). Three loops of C6.5 were simultaneously mutated by PM and the resulting gene repertoire cloned for display on the surface of phage. C6.5 mutants with sixfold higher affinity for c-erbB-2 ( $K_d = 2.4 \times 10^{-9}$  M) were selected from the library and aa within the loops important for modulation of affinity identified.

## RESULTS AND DISCUSSION

**(a) Selection of sites to be mutagenized and doping codons**

The immunoglobulin light-chain variable region ( $V_L$ ) of C6.5 is a member of the  $V_{\lambda 1}$  family, and could be modeled using the three dimensional structure of the  $V_{\lambda 1}$  domain of KOL (Marquart et al., 1980). L1 consists of nine aa, L2 of three aa and L3 of eight aa (Chothia and Lesk, 1987). The immunoglobulin heavy-chain variable region ( $V_H$ ) of C6.5 is derived from the DP73 germline gene of the  $V_{H5}$  family (Tomlinson et al., 1992) and could be modeled using the three-dimensional structure of the  $V_H$  domain of NC41 (Tulip et al., 1992). H1 consists of seven aa, H2 of six aa and H3 of 17 aa (Chothia et al., 1992). Thus, the loops consist of a total of 50 aa, too large a sequence space to search simultaneously, even using PM. L2 was excluded from PM, since it is the loop that least frequently contains aa which contact Ag (Wilson and Stanfield, 1993). H1 was excluded because three of the seven aa ( $G^{26}$ ,  $F^{27}$  and  $F^{29}$ ) have structural roles and the aa at these positions are generally conserved in  $V_H$  domains (Chothia and Lesk, 1987; Chothia et al., 1992). H3 was excluded from PM due to its length. The remaining three loops (L1, L3 and H2) were selected for randomization by PM. All eight aa of L3 were subjected to PM, as were all six aa of H2. Five C-terminal aa of

L1 (28–32, Kabat numbering; Kabat et al., 1987) were subjected to PM. Residues 26 to 27b were excluded from PM, since they are relatively conserved in Ab structures and are more constrained by framework contacts.

We subjected 19 aa to PM. PM-CAD was used to select mutation frequencies and doping codons, and to compute nt mixtures for oligo synthesis (Balint and Larrick, 1993). The library was designed so that the most abundant sequences contained five non-parental aa. Thus the frequency of a non-parental aa at each site is 0.26 (5/19), with approx. 80% of the library containing between two and seven non-parental aa. At each position,

alternative aa sets ranged from ten to 19 aa encoded by 12 to 32 codons (Tables I and II).

### (b) Construction and characterization of the PM phage Ab library

The PM randomized C6.5 *scFv* gene repertoire was assembled from three overlapping polymerase chain reaction (PCR) fragments consisting of a portion of the wt *scFv* gene and the mutagenized L1, L3, or H2 (Fig. 1). The N-terminal fragment (PM1) extended from upstream from an *Sfi*I site to approx. 40 nt beyond the mutagenized region of H2, which was encoded by the downstream

TABLE I

Nucleotide mixtures for PM of 19 aa located within  $V_{\lambda}$ CDR1,  $V_{\lambda}$ CDR3 and  $V_H$ CDR2 of C6.5 *scFv*<sup>a</sup>

		$V_{\lambda}$ CDR1														
aa <sup>b</sup> :	$I^{28}$			$G^{29}$			$N^{30}$			$N^{31}$			$Y^{32}$			
nt <sup>c</sup>	N	N	K	N	N	T	N	N	K	N	N	K	N	N	K	
A	91	3	0	5	5	0	91	91	0	91	91	0	3	91	0	
C	3	3	0	5	5	0	3	3	0	3	3	0	3	3	0	
G	3	3	10	85	85	0	3	3	10	3	3	10	3	3	10	
T	3	91	90	5	5	100	3	3	90	3	3	90	91	3	90	

		$V_{\lambda}$ CDR3																						
aa:	$W^{91}$			$D^{92}$			$D^{93}$			$S^{94}$			$L^{95}$			$S^{95a}$			$G^{95b}$			$W^{96}$		
nt	D	D	K	N	N	K	N	N	K	N	V	T	D	D	K	N	V	T	N	N	T	D	D	K
A	5	5	0	3	91	0	3	91	0	5	0	0	5	5	0	5	7	0	5	5	0	5	5	0
C	0	0	0	3	3	0	3	3	0	5	86	0	0	0	0	5	86	0	5	5	0	0	0	0
G	5	90	90	91	3	10	91	3	10	5	7	0	5	5	90	5	7	0	85	85	0	5	90	90
T	90	5	10	3	3	90	3	3	90	85	0	100	90	90	10	85	0	100	5	5	100	90	5	10

		$V_H$ CDR2																
aa:	$Y^{52}$			$P^{52a}$			$G^{53}$			$D^{54}$			$S^{55}$			$D^{56}$		
nt	N	N	K	N	N	T	N	N	T	N	N	K	N	V	T	N	N	K
A	3	91	0	5	5	0	5	5	0	3	91	0	5	7	0	3	91	0
C	3	3	0	85	85	0	5	5	0	3	3	0	5	86	0	3	3	0
G	3	3	10	5	5	0	85	85	0	91	3	10	5	7	0	91	3	10
T	91	3	90	5	5	100	5	5	100	3	3	90	85	0	100	3	3	90

<sup>a</sup> **Methods:** PM-CAD was used for calculating nt mixtures for synthesis of three spiked oligos, as described in Balint and Larrick (1994), based on the most prevalent mutant having five non-parental aa. The aa encoded by the oligos are located within one of three CDRs ( $V_{\lambda}$ CDR1,  $V_{\lambda}$ CDR3, or  $V_H$ CDR2) chosen for mutagenesis. Approx. 80% of the library should have 2–7 aa changes per *scFv*.

<sup>b</sup> The CDR aa selected for mutagenesis are listed horizontally in order according to Kabat number (Kabat et al., 1987) and the parental aa at each position is indicated in single-letter code. The CDR subjected to PM is also indicated above the first aa position randomized.

<sup>c</sup> Beneath each CDR position is the doping codon in IUB nt code (J. Biol. Chem. 261 (1986) 13): D=A, G, or T; K=G or T; N=A, C, G, or T; V=A, C, or G. Beneath each position in the doping codon is listed the proportion of each nt in mol%, computed as described in Balint and Larrick (1994) and used for synthesis of spiked oligos. The nt are listed down the left margin.

TABLE II  
 Predicted frequencies of parental and non-parental aa at C6.5 scFv aa positions subjected to PM<sup>a</sup>

	V <sub>λ</sub> CDR1										V <sub>λ</sub> CDR3										V <sub>H</sub> CDR2									
	I <sup>28</sup>	G <sup>29</sup>	N <sup>30</sup>	N <sup>31</sup>	Y <sup>32</sup>	W <sup>91</sup>	D <sup>92</sup>	D <sup>93</sup>	S <sup>94</sup>	L <sup>95</sup>	S <sup>95a</sup>	G <sup>95b</sup>	W <sup>96</sup>	Y <sup>52</sup>	P <sup>52a</sup>	G <sup>53</sup>	D <sup>54</sup>	S <sup>55</sup>	D <sup>56</sup>											
Non-polar aa																														
G	0.10	73.68	0.10	0.10	0.10	4.36	2.91	2.91	0.33	0.23	0.33	73.68	4.36	0.10	0.22	73.68	2.91	0.33	2.91											
A	0.10	4.05	0.10	0.10	0.10	2.91	2.91	4.10	4.10	4.10	4.10	4.05	2.91	0.10	4.05	4.05	2.91	4.10	2.91											
P	0.10	0.22	0.10	0.10	0.10	0.10	0.10	4.10	4.10	4.10	0.22	0.22	0.10	0.10	73.68	0.22	0.10	4.10	0.10											
V	2.91	4.05	0.10	0.10	0.10	0.23	2.91	2.91	4.37	4.37	4.05	0.23	0.23	0.10	0.22	4.05	2.91	4.10	2.91											
L	3.19	0.22	0.11	0.11	0.38	3.94	0.11	0.11	73.68	73.68	0.22	3.94	0.22	0.38	4.05	0.22	0.11	0.11	0.11											
I	73.68	0.22	2.63	2.63	0.09	0.02	0.09	0.09	0.42	0.42	0.22	0.02	0.02	0.09	0.22	0.22	0.09	0.09	0.09											
M	7.89	0.22	0.28	0.28	0.01	0.21	0.01	0.01	3.95	3.95	0.21	0.21	0.21	0.01	0.01	0.01	0.01	0.01	0.01											
F	2.63	0.22	0.09	0.09	2.63	0.42	0.09	0.09	7.90	7.90	0.22	0.42	0.42	2.63	0.22	0.22	0.09	0.09	0.09											
W	0.01	0.28	0.01	0.01	0.28	73.68	0.01	0.01	3.95	3.95	73.68	0.28	73.68	0.28	0.22	0.22	0.01	0.01	0.01											
Polar aa																														
S	2.73	4.27	2.73	2.73	3.00	0.42	0.19	0.19	74.01	0.02	74.01	4.27	0.42	3.00	4.27	4.27	0.19	74.01	0.19											
C	0.09	4.05	0.09	0.09	2.63	7.90	0.09	0.09	6.10	0.42	6.10	4.05	7.9	2.63	0.22	4.05	0.09	6.10	0.09											
T	2.91	0.22	2.91	2.91	0.10	0.10	0.10	0.10	4.10	4.10	4.10	0.22	0.10	0.10	4.05	0.22	0.10	4.10	0.10											
Q	2.63	0.22	73.68	73.68	2.63	0.02	2.63	2.63	0.33	0.02	0.33	0.22	0.02	2.63	0.22	0.22	2.63	0.33	2.63											
N	0.02	0.56	0.56	0.56	8.17	3.94	0.56	0.56	3.95	3.95	3.94	3.94	3.94	8.18	0.56	0.56	0.56	3.95	0.56											
H	0.09	0.22	2.63	2.63	2.63	2.63	2.63	2.63	0.33	0.33	0.33	0.22	2.63	2.63	0.22	0.22	2.63	0.33	2.63											
Y	0.09	0.22	2.63	2.63	73.68	0.42	2.63	2.63	6.10	0.42	6.10	0.22	0.42	73.68	0.22	0.22	2.63	6.10	2.63											
Charged aa																														
D	0.09	4.05	2.63	2.63	2.63	0.02	73.68	73.68	0.33	0.02	0.33	4.05	0.02	2.63	0.22	4.05	73.68	0.33	73.68											
E	0.01	0.28	0.28	0.28	0.28	0.21	7.90	7.90	0.21	0.21	0.21	0.21	0.21	0.28	0.21	0.21	7.90	0.28	7.90											
K	0.08	7.89	7.89	7.89	0.28	0.21	2.28	2.28	0.21	0.21	0.21	0.21	0.21	0.28	0.21	0.21	0.28	0.28	0.28											
R	0.38	4.05	0.38	0.38	0.11	3.94	0.11	0.11	0.33	0.21	0.33	4.05	3.94	0.11	4.05	4.05	0.11	0.33	0.11											

<sup>a</sup> **Methods:** The frequency of parental and non-parental aa at each position subjected to PM was calculated from the nt frequencies listed in Table I. The parental aa is listed across the top of each column, using single-letter aa code, with the position number in the V<sub>H</sub> or V<sub>λ</sub> domain according to Kabat et al. (1987). The CDR subjected to PM is indicated above the first aa position randomized. aa resulting from PM are listed down the left margin and are grouped into three categories, non-polar aa, polar aa and charged aa. Predicted frequencies of aa are indicated by numerals, with empty spaces representing aa substitutions with a predicted frequency of zero. PM results in a preference for conservative aa substitutions.

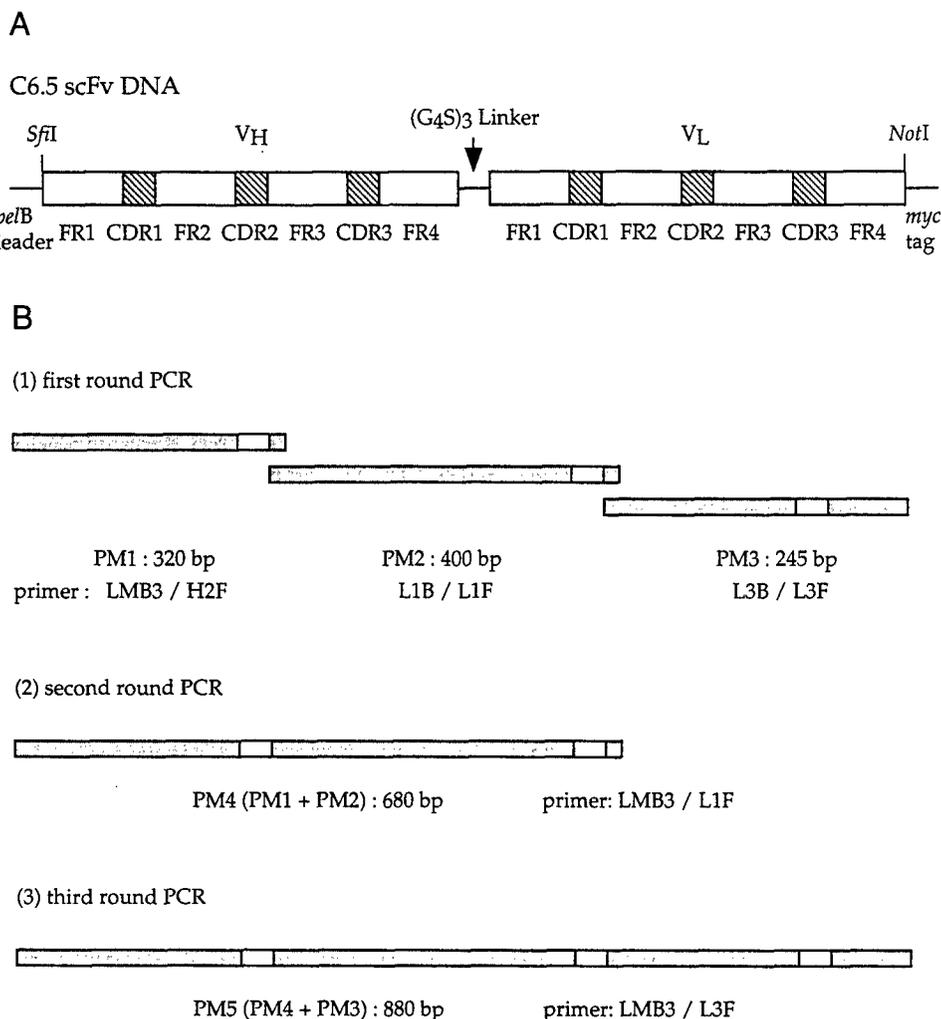


Fig. 1. Construction of C6.5 scFv PM phage Ab library. The C6.5 scFv construct is shown in panel A with a *pelB* leader sequence for secretion into the periplasm of *E. coli*, the V<sub>H</sub> and V<sub>L</sub> of C6.5 joined by the (G<sub>4</sub>S)<sub>3</sub> linker, and the myc tag for detection of the produced ScFv (open boxes indicate the framework regions (FR) and hatched boxes the CDRs of V<sub>H</sub> and V<sub>L</sub>). In section B is shown the PCR assembly process used to create an ScFv gene repertoire where 19 aa located within CDR1 and CDR3 of the V<sub>L</sub> gene and CDR2 of the V<sub>H</sub> gene were subjected to PM (shaded boxes indicate unmutated DNA segments and open boxes CDRs subjected to PM). (1) Pairs of oligo primers (described in Table III) were used to amplify three gene segments (PM1, PM2 and PM3) from C6.5 plasmid DNA (10 ng/μl) (Schier et al., 1995a). Oligos H2F, L1F and L3F encoded the 19-aa loop residues subjected to PM (open boxed area in gene segments of B). (2) Gel-purified fragments PM1 and PM2 (200 ng each) were used as template for the next PCR round to produce PM4. PM1 and PM2 were first cycled seven times without primer (94°C for 30 s, 60°C for 5 s, 40°C for 5 s (RAMP 5 s) and 72°C for 60 s) to join the fragments together. Primers were then added (LMB3 and L1F, 25 pmol each) and the reaction mixture subjected to 30 cycles of amplification (94°C for 30 s, 50°C for 30 s and 72°C for 60 s). (3) Splicing and amplification were repeated to join purified PM4 and PM3 using the primers LMB3 and L3F, to create the fragment PM5. PM5 was reamplified using the primers LMB3 and HuJ<sub>2</sub>-3ForNot to introduce a *NotI* restriction site at the 3' end of the ScFv gene repertoire. The resulting ScFv gene repertoire was digested with *SfiI* + *NotI*, gel purified and ligated into pCANTAB5E (Pharmacia) digested with *SfiI* + *NotI*. Aliquots of the ligation mixture were electroporated into electrocompetent *E. coli* TG1 (Gibson, 1984).

primer H2F (Table III and Fig. 1). The second fragment (PM2) extended from the C terminus of HCDR2 to approx. 40 nt downstream from the mutagenized region of L1, which was encoded by the downstream primer L1F. The third fragment (PM3) extended from the C terminus of LCDR1 to approx. 40 nt downstream from the mutagenized region of L3, which was encoded by the downstream primer L3F. The three gene fragment repertoires were spliced together using PCR and the resulting scFv gene repertoire cloned into the phage display vector

pCANTAB5E (Pharmacia). After transformation of *E. coli* TG1 (Gibson, 1984), a library of  $1.0 \times 10^6$  clones was obtained. By PCR screening of colonies (Gussow and Clackson, 1989), 88% of the clones contained a full length scFv gene, giving an effective library size of  $8.8 \times 10^5$ . The V<sub>H</sub> and V<sub>L</sub> genes of eight unselected scFv were sequenced to determine the frequency and location of mutations in the library (Table VI). Each scFv gene averaged four single-aa substitutions in the three regions subjected to PM, with a range of three to six substitutions. In addition,

TABLE III

Sequences of primers used for construction of PM phage Ab library<sup>a</sup>

Primer	Oligo sequence <sup>b</sup>
LMB3	5'-CAGGAAACAGCTATGAC'
HuJλ2-3ForNot	5'-GAGTCATTCTCGACTTGC GGCCGCACCTAGGACGGTCAGCTTGGTCCC
L1B	5'-ACCAAATACAGCCCGTCCTTCCAAGGCCAG
L3B	5'-GTATCCTGGTACCAGCAGCTCCCAGGAAC
L1F	5'-GAGTTTGGGGGCTGTTCTCTGGGAGCTGCTGGTACCAGGATAC1,2,8,1,2,2,1,2,2,A,6,6,1,8,2,GTTGGAGCAGCTTCC
L3F	5'-CGATGCGGCCGCACCTAGGACGGTCAGCTTGGTCCCTCCGCCGAACAC11,10,9,A,6,6,A,4,5,11,9,9,A,4,5,1,2,3,1,2,3,11,10,9,TGCTGCACAG
H2F	5'-GATGGTGACCTGGCCTTGGGAAGGACGGGCTGTATTGGT1,2,3,A,4,5,1,2,3,A,6,6,A,7,7,1,2,8,GATGA GCCCCATGACTC
Nucleotide mixtures <sup>c</sup>	
1: A(0.9), C(0.1); 2: T(0.91), C, A and G (0.03); 3: C(0.91), T, A and G(0.03); 4: G(0.86), T and C(0.07); 5: A(0.85), T, C and G(0.05); 6: C(0.85), T, A and G, (0.05); 7: G(0.85), T, C and A(0.05); 8: A(0.91), T, C, G(0.03); 9: A(0.9), T, C(0.05); 10: C(0.9), A, T(0.05); 11: C(0.9), A(0.1)	

<sup>a</sup> **Methods:** Oligos L1F, L3F and H2F were synthesized by Keystone Laboratories (Palo Alto, CA, USA) and the remainder of the oligos were synthesized by Genset (La Jolla, CA, USA).

<sup>b</sup> Oligos used to PCR amplify C6.5 DNA fragments PM1 to PM5, as shown in Fig. 1. The numbers 1 to 11 indicate one of eleven nt mixtures (shown below) used at that oligo position.

<sup>c</sup> Nucleotide mixtures used to generate the oligos L1F, L3F and H2F. In parentheses are the fractions of nt (A, C, G and T) comprising each of the eleven nt mixtures.

an average of 0.9 substitutions per *scFv* were observed outside of the regions subjected to PM, presumably due to PCR errors.

### (c) Selection and characterization of higher-affinity *scFv*

The PM phage Ab library was subjected to four rounds of selection in solution on biotinylated c-erbB-2 extracellular domain (c-erbB-2 ECD), starting with an Ag concentration of  $4.0 \times 10^{-8}$  M and decreasing to  $1.0 \times 10^{-11}$  M (Table IV). This selection approach uses limiting Ag concentrations in the latter rounds to drive affinity-based selection, while the high Ag concentration in early rounds ensures the capture of rare binders (Schier et al., 1996). Prior to selection, only 3/92 *scFv* bound c-erbB-2 ECD by enzyme-linked immunosorbent assay (ELISA), while after three and four rounds of selection, virtually all *scFv* bound c-erbB-2 ECD (Table IV). The dissociation rate constant (Karlsson et al., 1991) was determined on unpurified *scFv* present in bacterial periplasm for 20 ELISA positive clones from the third and fourth rounds of selection using surface plasmon resonance (SPR) in a BIAcore (Jönsson et al., 1991). After three rounds of selection, three of 20 *scFv* (12%) had a  $k_{off}$  lower than the wt *scFv*, while after four rounds of selection, 10/20 *scFv* (50%) had a lower  $k_{off}$ . All 13 *scFv* with a lower  $k_{off}$  were sequenced, subcloned into plasmid pUC119Hismyc (Schier et al., 1995) and purified by immobilized metal chelate chromatography, followed by gel filtration to remove any *scFv* aggregates. Affinities were determined for each *scFv* by SPR in a BIAcore (Karlsson et al., 1991). Two of the three *scFv* isolated after the third round of selection were not of higher

TABLE IV

Frequency of binding *scFv* and percent of binding *scFv* with lower  $k_{off}$  than the parental *scFv*<sup>a</sup>

Round of selection	Ag concentration ( $\times 10^{-9}$ M)	ELISA-positive clones <sup>b</sup>	% clones with lower $k_{off}$ than parental <i>scFv</i> <sup>c</sup>
1	40	ND	ND
2	1	ND	ND
3	0.1	92/92	12
4	0.01	91/92	50

<sup>a</sup> **Methods:** Phage were subjected to four rounds of selection using decreasing concentrations of biotinylated c-erbB-2 ECD. Phage were rescued, incubated with biotinylated c-erbB-2 ECD, captured with streptavidin-coated M280 Dynabeads (Dyna), the beads washed, and the washed beads with bound phage used to infect *E. coli* TG1, exactly as described in Schier et al. (1996). Phage were prepared for the next round of selection, exactly as described in Schier et al. (1996).

<sup>b</sup> *scFv* was produced (De Bellis and Schwartz, 1990) by 96 randomly selected clones in microtitre plates (Marks et al., 1991) and the supernatant harvested and used for ELISA on biotinylated c-erbB-2 ECD captured on avidin-coated Immulon 4 microtitre plates (Dynatech) exactly as described in Schier et al. (1996). Binding was detected using an anti-E tag antibody (Pharmacia), which recognizes the E-tag at the C-terminus on the *scFv*, followed by anti-mouse Fc-HRP. ND, not determined.

<sup>c</sup> *scFv* was produced by 20 clones from the third and 20 clones from the fourth round of selection, the periplasm harvested (Breitling et al., 1991) and dialyzed overnight against HBS.  $k_{off}$  was determined on the dialyzed periplasmic fraction using SPR in a BIAcore (Jönsson et al., 1991), exactly as described in Schier et al. (1996).

affinity than the wt *scFv*, while the third had an affinity threefold higher than wt (Table V). All ten *scFv* from the fourth round of selection had higher affinity than the wt *scFv*, with the best clone (C61.M6) having a sixfold increase in affinity ( $2.4 \times 10^{-9}$  M). The results confirm

TABLE V  
Kinetics of selected scFv subjected to PM<sup>a</sup>

scFv clone	$k_{on}$ ( $\times 10^5$ M <sup>-1</sup> s <sup>-1</sup> )	$k_{off}$ ( $\times 10^{-3}$ s <sup>-1</sup> )	$K_d$ (M)
C6.5	4.0 $\pm$ 0.1	6.3 $\pm$ 0.05	1.6 $\times 10^{-8}$
PM1	3.9 $\pm$ 0.34	7.4 $\pm$ 0.12	1.9 $\times 10^{-8}$
PM2	5.5 $\pm$ 0.1	10.5 $\pm$ 0.10	1.9 $\times 10^{-8}$
PM3	5.6 $\pm$ 0.5	2.9 $\pm$ 0.1	5.2 $\times 10^{-9}$
PM4	10.0 $\pm$ 0.5	4.5 $\pm$ 0.09	4.5 $\times 10^{-9}$
PM5	4.6 $\pm$ 0.08	1.7 $\pm$ 0.09	3.7 $\times 10^{-9}$
PM6	6.6 $\pm$ 0.37	1.6 $\pm$ 0.03	2.4 $\times 10^{-9}$
PM7	4.9 $\pm$ 0.06	2.1 $\pm$ 0.09	4.3 $\times 10^{-9}$
PM8	4.4 $\pm$ 0.33	1.3 $\pm$ 0.11	2.9 $\times 10^{-9}$
PM9	7.7 $\pm$ 0.24	5.1 $\pm$ 0.09	6.6 $\times 10^{-9}$
PM10	8.4 $\pm$ 0.1	5.9 $\pm$ 0.11	7.0 $\times 10^{-9}$
PM11	7.7 $\pm$ 0.5	4.8 $\pm$ 0.09	6.2 $\times 10^{-9}$
PM12	5.7 $\pm$ 0.17	1.9 $\pm$ 0.13	3.3 $\times 10^{-9}$
PM13	8.3 $\pm$ 0.5	4.3 $\pm$ 0.1	5.2 $\times 10^{-9}$

<sup>a</sup> **Methods:**  $k_{on}$  and  $k_{off}$  were measured on purified scFv using SPR in a BIAcore (Jønsson et al., 1991), as described in Schier et al. (1996). 1400 resonance units c-erbB-2 ECD (90 kDa) were coupled to a CM5 sensorchip (Jønsson et al., 1991) and  $k_{on}$  and  $k_{off}$  measured under continuous flow of 5  $\mu$ l/min using an scFv concentration ranging from 50 to 800 nM.  $k_{on}$  was determined from a plot of  $\ln(dR/dt)/t$  vs. concentration (Karlsson et al., 1991).  $k_{off}$  was determined from the dissociation part of the sensorgram at the highest concentration of scFv analyzed (Karlsson et al., 1991). During dissociation, rebinding was excluded by comparing  $k_{off}$  with the  $k_{off}$  determined in the presence of 500 nM c-erbB-2 ECD in HBS during the dissociation phase.  $K_d$  was calculated as  $k_{off}/k_{on}$ .

the effectiveness of the selection approach to enrich for higher affinity scFv and BIAcore screening to identify higher affinity scFv. Only two of 13 scFv purified did not have an improved affinity. Both of these scFv were from the third round of selection. The affinity of C6PM6 ( $2.4 \times 10^{-9}$  M) compares favorably to the affinity of murine Ab produced against the same Ag using conventional hybridoma technology (Carter et al., 1992; Adams et al., 1993).

#### (d) Location of mutations in selected clones

Sequence analysis of higher affinity scFv indicated that substitutions occurred at 10/19 (53%) of the positions, with 21/28 substitutions occurring at only four aa positions, two in H2, and one each in L1 and L3 (Table VI). Thus, PM identified a subset of 'functional' aa whose mutation results in increased affinity. All but one of these ten aa ( $V_{\lambda}L^{95}$ ) appear to have solvent accessible side chains in our C6.5 model. In contrast, two aa ( $V_{\lambda}N^{30}$  and  $V_HY^{52}$ ) with solvent exposed side chains are 100% conserved, suggesting these are 'functional' aa which are critical for recognition.

The majority (7/9) of the conserved aa, however, appear to have a structural role in the variable domain, either in maintaining the main chain conformation of the loop, or in packing on the  $V_H$  domain. In the  $V_{\lambda}$  domain,

aa  $I^{28}$ ,  $G^{29}$ ,  $W^{91}$  and  $D^{92}$  are present in both C6.5 and KOL (Marquart et al., 1980), consistent with a structural role. The side chain of  $I^{28}$  is buried deep in the core of the  $V_{\lambda}$  domain between hydrophobic aa 25, 33 and 71, and is a major determinant of the main chain conformation of L1 (Chothia and Lesk, 1987). In the model of C6.5,  $V_{\lambda}G^{29}$ ,  $V_{\lambda}G^{95b}$  and  $V_HG^{53}$  are in turns and  $V_{\lambda}W^{91}$  and  $V_{\lambda}W^{96}$  pack against the  $V_H$  domain at the  $V_H$ - $V_L$  interface. Hydrogen bonds between  $V_{\lambda}D^{92}$  and  $V_{\lambda}S^{27a}$  and  $V_{\lambda}N^{27b}$  bridge L3 and L1 to stabilize the L3 and L1 conformations. The results suggest that even conservative substitution of aa known to have a structural role does not produce higher affinity Ab. Thus, efficient in vitro evolution of proteins could be achieved by reducing the sequence space that requires scanning by homology modeling or sequence alignments of members of structurally related families.

#### (e) Comparison to other mutagenesis techniques used with phage display

The increase in affinity achieved by PM of C6.5 is virtually identical to that achieved by H or L chain shuffling C6.5 ( $2.4 \times 10^{-9}$  M and  $3.4 \times 10^{-9}$  M, respectively) (Schier et al., 1996). The sixfold increase in affinity is also comparable to the eightfold increase in affinity achieved using phage display and sequential mutagenesis of  $V_H$ CDR1 and  $V_H$ CDR3 of an anti-gp120 Fab (Barbas et al., 1994), or the three to sixfold increase in affinity achieved from a single mutagenic phage hormone library (Lowman and Wells, 1993). Only PM, however, permitted the scanning of a much larger sequence space, resulting in identification of two aa subsets: non-conserved functional aa which modulate affinity, and conserved aa, the majority of which have a structural role. To obtain even higher affinity Ab, functional aa that modulate affinity could be selected for more thorough scanning, using a higher mutagenic rate (Delagrave and Youvan, 1993). PM should also prove useful for modifying function in other proteins that belong to structurally related families.

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TABLE VI

Deduced aa sequences of CDRs of C6.5 and C6.5 clones from the PM phage Ab library<sup>a</sup>

Residue number <sup>b</sup> :	V <sub>H</sub> CDR2	V <sub>λ</sub> CDR1	V <sub>λ</sub> CDR3
C6.5	5 5 5 6 6 0 2 a 3 0 5 L I Y P G D S D T K Y S P S F Q G	2 2 2 3 3 4 7 a b 8 0 4 S G S S S N I G N N Y V S	8 9 9 9 5 a b 7 A A W D D S L S G W V
3rd round selection			
PM1 <sup>c</sup>	- - - - - S - - - - -	- - - - - M D - - - - -	- - - - - T - - - - -
PM2	F - - - - -	- - - - - K - - - - -	- - - - - E - W T - - - - -
PM3	S - - - - N Y - - - - -	- - - - -	- - - - - Y - - - - -
4th round selection			
PM4	- - - - -	- - - - -	- - - - -
PM5	- - - - - Y G - - - - -	- - - - - T - - - - -	- - - - - Y - - - - -
PM6	- - - - - Y G - - - - -	- - - - - K - - - - -	- - - - - Y - - - - -
PM7	- - - - -	- - - - -	- - - - - H - - - - -
PM8	- - - A - - - A - - - - -	- - - - -	- - - - - Y - - - - -
PM9	- - - - -	- - - - - K - - - - -	- - - - - Y - - - - -
PM10	- - - - -	- - - - - D - - - - -	- - - - - A - Q Y - - - - -
PM10	- - - - -	- - - - - S - - - - -	- - - - - A - - - - -
PM12	- - - - -	F - - - - -	- - - - - E - - - - -
PM13	- - - - - Y G - - - - -	- - - - - K - - - - -	- - - - - Y - - - - -
Unselected clones			
1	- - - - S N - - - - -	- - - - - F - - - - -	- - - - -
2 <sup>d</sup>	- - - - -	- - - - - S - - - - -	- - - * E - - - C - - - - -
3	- - F - - V T E - - - - -	- - - - - N - - - - -	- - - Y - - - - -
4 <sup>d</sup>	- - * - - N - - - - -	- - - - -	- - - - - C - C - - - -
5	- - - - E F N - - - - -	- - - - -	- - - E F - - - - -
6	- - - - -	- - - - -	- - - - A - T C - - - -
7	- - - N - V - - - - -	- - - - -	- - R - - - A - - - -
8	- - - - D - C - - - - -	- - - - -	- - - - - A - - - -

<sup>a</sup> **Methods:** The DNA of eight unselected scFv and c-erbB-2-binding scFv from the third and fourth round of selection was sequenced (Sanger et al., 1977), the aa sequence deduced and the sequences aligned with the deduced aa sequence of V<sub>H</sub>CDR2, V<sub>λ</sub>CDR1 and V<sub>λ</sub>CDR3 of C6.5. Underlined aa are those subjected to PM. Sequences have been deposited with GenBank, accession Nos. U38244 and U38323-U38347.

<sup>b</sup> Residue numbers (50–65 for V<sub>H</sub>CDR2, 24–34 for V<sub>λ</sub>CDR1 and 89–97 for V<sub>λ</sub>CDR3) are according to Kabat et al. (1987).

<sup>c</sup> Dashes indicate aa sequence identity with the original clone C6.5.

<sup>d</sup> Asterisks represent stop codons.

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Appendix 5

Schier R, McCall A, Adams GP, Marshall K, Yim M, Merritt H, Crawford RS, Weiner LM, Marks JD. Isolation of high affinity anti-c-erbB-2 single-chain Fv by molecular evolution of the complementarity determining regions in the center of the antibody combining site. *J. Mol. Biol.* 263: 551-567, 1996.

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## Isolation of Picomolar Affinity Anti-c-erbB-2 Single-chain Fv by Molecular Evolution of the Complementarity Determining Regions in the Center of the Antibody Binding Site

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We determined the extent to which additional binding energy could be achieved by diversifying the complementarity determining regions (CDRs) located in the center of the antibody combining site of C6.5, a human single-chain Fv (scFv) isolated from a non-immune phage library which binds the tumor antigen c-erbB-2. CDR3 of the light ( $V_L$ ) and heavy ( $V_H$ ) chain variable region of C6.5 were sequentially mutated, the mutant scFv displayed on phage, and higher affinity mutants selected on antigen. Mutation of  $V_L$  CDR3 yielded a scFv (C6ML3-9) with a 16-fold lower  $K_d$  ( $1.0 \times 10^{-9}$  M) than C6.5. Due to its' length of 20 amino acids, four  $V_H$  CDR3 libraries of C6ML3-9 were constructed. The greatest increase in affinity from a single library was ninefold ( $K_d = 1.1 \times 10^{-10}$  M). Combination of mutations isolated from separate  $V_H$  CDR3 libraries yielded additional ninefold decreases in  $K_d$ , resulting in a scFv with a 1230-fold increase in affinity from wild-type C6.5 ( $K_d = 1.3 \times 10^{-11}$  M). The increase in affinity, and its absolute value, are comparable to the largest values observed for antibody affinity maturation *in vivo* or *in vitro* and indicate that mutation of  $V_L$  and  $V_H$  CDR3 may be a particularly efficient means to increase antibody affinity. This result, combined with the location of amino acid conservation and substitution, suggests an overall strategy for *in vitro* antibody affinity maturation. In addition, the affinities and binding kinetics of the single-chain Fv provide reagents with potential tumor targeting abilities not previously available.

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**Keywords:** c-erbB-2; single-chain Fv; antibody phage display; affinity maturation; BIAcore

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### Introduction

Antibody based cancer therapy is limited by the properties of antibodies derived from conventional hybridoma technology (reviewed by Riethmueller

*et al.*, 1993). IgG are large molecules (150 kDa) which diffuse slowly into tumors (Clauss & Jain, 1990) and are slowly cleared from the circulation, resulting in poor tumor:normal organ ratios (Sharkey *et al.*, 1990). Smaller single-chain Fv

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Abbreviations used: AMP, ampicillin; c-erbB-2 ECD, c-erbB-2 extracellular domain; CDR, complementarity determining region; ELISA, enzyme linked immunosorbent assay; FACS, fluorescence activated cell sorter; FR, framework region; Glu, glucose; HBS, Hepes buffered saline (10 mM Hepes, 150 mM NaCl, pH 7.4); IMAC, immobilized metal affinity chromatography;  $k_{on}$ , association rate constant;  $k_{off}$ , dissociation rate constant; MPBS, skimmed milk powder in PBS; MTPBS, skimmed milk powder in TPBS; PBS, phosphate buffered saline (25 mM NaH<sub>2</sub>PO<sub>4</sub>, 125 mM NaCl, pH 7.0); PCR, polymerase chain reaction; RU, resonance units; scFv, single-chain Fv fragment; TPBS, 0.05% (v/v) Tween 20 in PBS; SPR, surface plasmon resonance;  $V_L$ , immunoglobulin kappa light chain variable region;  $V_{L\lambda}$ , immunoglobulin lambda light chain variable region;  $V_L$ , immunoglobulin light chain variable region;  $V_H$ , immunoglobulin heavy chain variable region; wt, wild-type.



(PCR) screening revealed that 30 of 30 randomly selected colonies had full length insert and diversity was confirmed by sequencing the V<sub>L</sub> CDR3 of ten unselected clones (results not shown). Prior to selection, 5/92 clones selected at random expressed scFv which bound c-erbB-2 ECD by enzyme linked immunosorbent assay (ELISA). After both the third and fourth rounds of selection, 92/92 clones bound c-erbB-2 ECD by ELISA.

The C6VLCDR3 library was selected using decreasing concentrations of biotinylated c-erbB-2 ECD, as described by Schier *et al.* (1996b). A relatively high antigen concentration ( $4.0 \times 10^{-8}$  M) was used for the first round to capture rare or poorly expressed phage antibodies. The antigen concentration was then decreased 40-fold for the second round ( $1.0 \times 10^{-9}$  M), and decreased a further tenfold each of the subsequent two rounds ( $1.0 \times 10^{-10}$  M, 3rd round;  $1.0 \times 10^{-11}$  M, 4th round). Reduction of the antigen concentration helps ensure that selection for higher affinity scFv occurs, rather than selection for scFv that express well on phage or are less toxic to *Escherichia coli* (Hawkins *et al.*, 1992; Schier *et al.*, 1996b). The optimal antigen concentration cannot be predicted *a priori*, due to variability in phage antibody expression levels and uncertainty regarding the highest affinities present in the mutant phage antibody library. Thus the choice of antigen concentration was guided by determining the percentage of binding phage present in the polyclonal phage preparation. After each round of selection, polyclonal phage were prepared and the concentration of binding phage determined by measuring the rate of binding to c-erbB-2 ECD under mass transport limited conditions using surface plasmon resonance (SPR) in a BIAcore (Schier & Marks, 1996). The percentage of binding phage was calculated by dividing the concentration of binding phage by the concentration of total phage as determined by infecting *E. coli*. If little or no change in the binding phage percentage was observed (as in these experiments), the antigen concentration was decreased significantly (at least tenfold) in the next round of selection. A large decrease in the percentage of binding phage, as we have observed during other selections (Schier & Marks, 1996), indicates that the selection should be repeated using a higher antigen concentration, or that the antigen concentration should not be decreased for the next round of selection.

#### Characterization of mutant scFv

To identify scFv with a lower  $K_d$  than wt scFv, apparent  $k_{off}$  was determined by SPR in a BIAcore on unpurified native scFv in bacterial periplasm (Schier *et al.*, 1996b). A total of 24 scFv from the third and fourth rounds of selection were ranked by  $k_{off}$ . After the third round of selection, 80% of scFv had a lower  $k_{off}$  than wt and after four rounds, 100% of scFv had a lower  $k_{off}$  than wt scFv. The 12 scFv with the lowest  $k_{off}$  from each of these rounds of

selection were sequenced and each unique scFv gene was subcloned for purification. scFv were purified by immobilized metal affinity chromatography (IMAC), followed by gel filtration to remove any dimeric or aggregated scFv. The  $k_{on}$  and  $k_{off}$  were determined by BIAcore, and the  $K_d$  calculated.

After the third round of selection, seven unique scFv were identified, all with higher affinity than wt scFv (Table 2). scFv had on average 1.8 amino acid substitutions/scFv, with a single substitution at residue 93 the most frequently observed mutation. This single amino acid substitution would have occurred with a frequency of 1/12,000 in the original library, assuming equal nucleotide coupling efficiency. The average scFv affinity was  $3.6 \times 10^{-9}$  M (4.4-fold increase), with the highest affinity  $2.6 \times 10^{-9}$  M (six-fold increase). After four rounds of selection, six scFv were identified, and none of these sequences were observed in the scFv sequenced from the third round (Table 2). For the selections reported above, binding phage were not specifically eluted, but rather were incubated with *E. coli*. We subsequently determined that when elutions are performed by incubating phage bound to antigen with *E. coli*, the phage probably must dissociate from antigen for infection to occur, leading to preferential selection of scFv with more rapid  $k_{off}$  (Schier & Marks, 1996). Steric hindrance, due to the size of paramagnetic beads, blocks the attachment of pIII on antigen bound phage to the f-pilus on *E. coli*. Repetition of the fourth round of selection using 100 mM HCl as eluent yielded an additional five scFv, including the highest affinity scFv obtained (C6ML3-9, Table 2). scFv from the fourth rounds of selection had on average 2.9 amino acid substitutions/scFv, with expected frequencies between 1/590,000 and 1/24,000,000 in the original library. The average scFv affinity after the fourth rounds of selection was  $1.9 \times 10^{-9}$  M (8.4-fold increase), with the highest affinity  $1.0 \times 10^{-9}$  M (16-fold increase). The results demonstrate the efficiency and importance of the selection and elution techniques for isolating very rare high affinity clones from a library.

#### Location of mutations in higher affinity scFv

Significant sequence variability (six different amino acids) was observed at residues 93, and 94, with less variability (three different amino acids) at residues 95 and 95a. Thus a subset of the randomized residues appear to be more important in modulating affinity. All but one of these four residues (V<sub>AL</sub>95) appear to have solvent accessible side-chains in our model of C6.5, which is based on the atomic structure of the Fab KOL (Figure 1). Three of the residues randomized (A89, W91, and G96) were 100% conserved in all mutants sequenced. Two additional residues (A90S and D92E) showed only a single conservative substitution. These conserved residues appear to have a structural role in the variable domain, either in maintaining the main-chain conformation of the

Table 2. Sequences, affinities and binding kinetics of scFv isolated from a library of C6.5 V<sub>L</sub> CDR3 mutants

Clone	F	V <sub>L</sub> CDR3 sequence	K <sub>d</sub> (10 <sup>-9</sup> M)	k <sub>on</sub> (10 <sup>5</sup> s <sup>-1</sup> M <sup>-1</sup> )	k <sub>off</sub> (10 <sup>-3</sup> s <sup>-1</sup> )
C6.5	0	8 9 9 9 5ab 7 <u>AAWDDSLSGWV</u>	16.0	4.0 ± 0.20	6.3 ± 0.06
A. Third round of selection					
C6ML3-7	1	----YAV----	2.6	6.5 ± 0.29	1.7 ± 0.09
C6ML3-2	2	----H-----	2.8	7.0 ± 0.24	2.0 ± 0.09
C6ML3-6	2	-S--Y-----	3.2	5.8 ± 0.43	1.9 ± 0.02
C6ML3-4	1	-S-EY--W---	3.4	3.8 ± 0.32	1.3 ± 0.13
C6ML3-5	4	----Y-----	3.7	5.2 ± 0.34	1.9 ± 0.08
C6ML3-3	1	-S--YR-----	3.8	5.5 ± 0.12	2.1 ± 0.05
C6ML3-1	1	----Y--W---	6.1	3.3 ± 0.07	2.0 ± 0.15
B. Fourth round of selection					
C6ML3-9 <sup>a</sup>	1	-S--YT-----	1.0	7.6 ± 0.20	0.76 ± 0.03
C6ML3-14 <sup>a</sup>	1	----P-W---	1.1	7.0 ± 0.20	0.77 ± 0.02
C6ML3-23 <sup>a</sup>	1	-S--H--W---	1.5	6.7 ± 0.41	1.7 ± 0.09
C6ML3-19 <sup>a</sup>	1	-S--RP-W---	1.5	6.6 ± 0.69	1.0 ± 0.02
C6ML3-12 <sup>a</sup>	2	----Y-R----	1.6	4.5 ± 0.16	0.72 ± 0.02
C6ML3-29	1	----GT-W---	1.7	12.9 ± 1.03	2.2 ± 0.02
C6ML3-15	1	----RP-W---	2.2	5.9 ± 0.81	1.3 ± 0.02
C6ML3-10	1	---E-P-Y---	2.3	6.1 ± 0.80	1.4 ± 0.02
C6ML3-13	1	----AT-W---	2.4	8.7 ± 0.98	2.1 ± 0.09
C6ML3-8	1	----HLRW---	2.6	6.4 ± 0.23	1.7 ± 0.15
C6ML3-11	1	----YA-W---	3.6	6.1 ± 0.15	2.2 ± 0.08

A, Mutants isolated after the third round of selection; B, Mutants isolated after the fourth round of selection. The entire V<sub>L</sub> CDR3 of C6.5 is shown, with the residues subjected to mutagenesis (89 to 95b) underlined. k<sub>on</sub> and k<sub>off</sub> were measured by SPR in a BIAcore, and the K<sub>d</sub> calculated. Dashes indicate sequence identity. F, frequency of isolated scFv. Numbering is according to Kabat *et al.* (1987).

<sup>a</sup> scFv obtained after elution with 100 mM HCl or 100 mM triethylamine.

loop, or in packing on the V<sub>H</sub> domain. Residues A89, W91, and D92 are identical in both C6.5 and KOL (Marquardt *et al.*, 1980), with conservative substitutions A90S and G96A observed at the other two positions in KOL, consistent with a structural role. In the model of C6.5, G95b is in a turn and A89, A90, and W91 are either buried or pack against the V<sub>H</sub> domain at the V<sub>H</sub>-V<sub>L</sub> interface (Figure 1). Hydrogen bonds between V<sub>λ</sub>D92 and V<sub>λ</sub>S27a and V<sub>λ</sub>N27b bridge L3 and L1 to stabilize the L3 and L1 conformations.

### Mutation of C6ML3-9 scFv V<sub>H</sub> CDR3

#### Library construction and selection

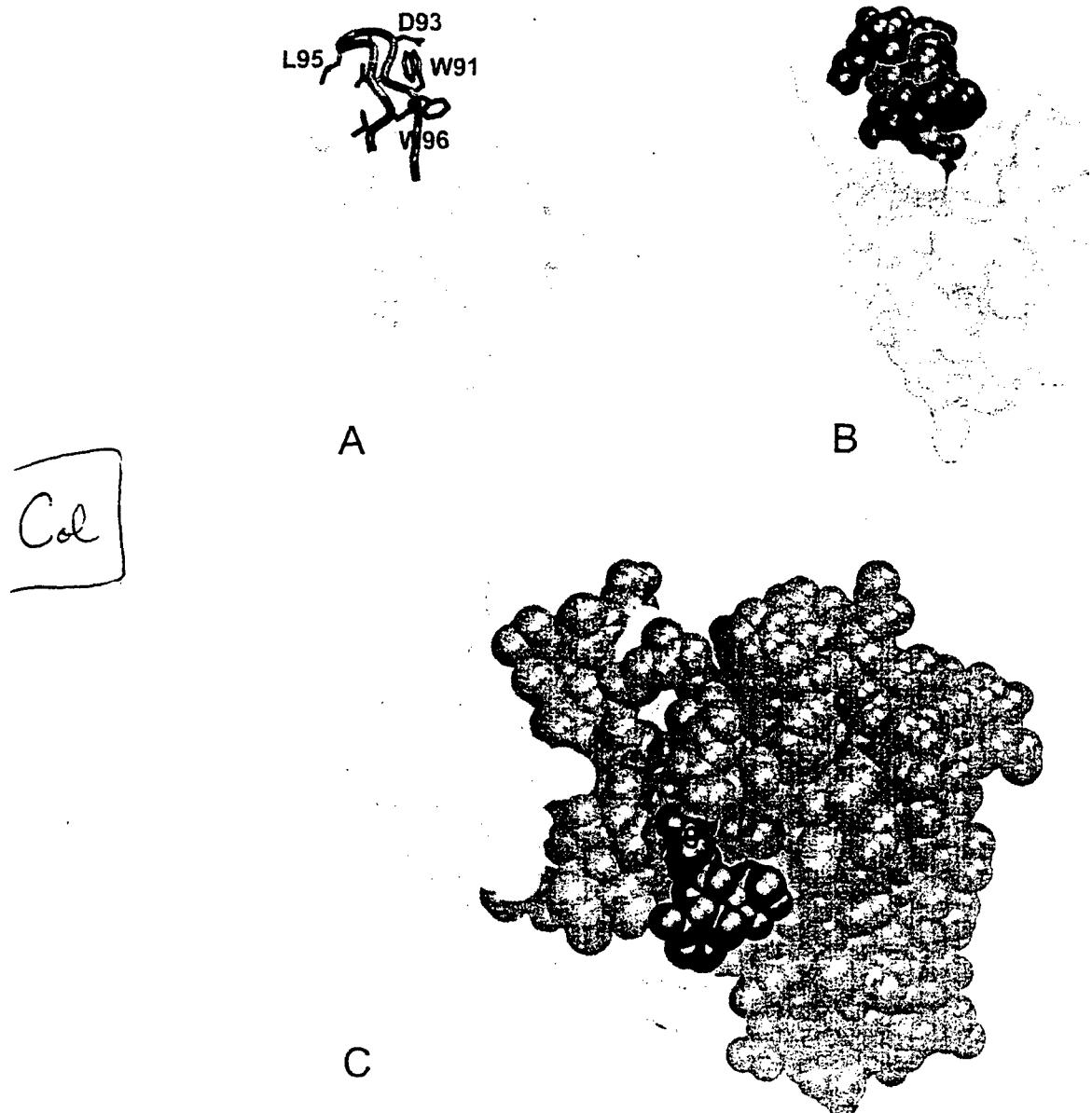
To further increase the affinity of C6.5, we chose to mutate the V<sub>H</sub> CDR3 of the highest affinity scFv (C6ML3-9, K<sub>d</sub> = 1.0 × 10<sup>-9</sup> M) isolated from the C6VLCDR3 library, rather than mutate C6.5 V<sub>H</sub> CDR3 independently and combine mutants. This sequential approach was taken since the kinetic effects of independently isolated antibody fragment mutations are frequently not additive (Yang *et al.*, 1995; Schier *et al.*, 1996b). Due to the length of the C6.5 V<sub>H</sub> CDR3 (20 amino acids), a high resolution functional scan was performed on C6.5 scFv in an attempt to reduce the number of amino acids subjected to mutation. Residues 95 to 99, 100a to 100d, and 100g to 102 were separately mutated to

alanine, and the K<sub>d</sub> of the mutated scFv determined. Residue 100f (alanine) was not studied. Residues 100 and 100e are a pair of cysteines separated by four amino acids. A homologous sequence in KOL (Marquardt *et al.*, 1980) results in a disulfide bond between the two cysteine residues and a four residue miniloop. Therefore the two cysteine residues were simultaneously mutated to serine. Results of the alanine scan are shown in Table 3. No detectable binding to c-erbB-2 ECD could be measured by BIAcore for C6.5H95A, C6.5W100hA, and C6.5E100jA. Three additional alanine mutants (G98A, Y100kA, and F1001A) yielded scFv with 20-fold to 100-fold higher K<sub>d</sub> than wt scFv. Substitution of the two cysteine residues by alanine (100, 100e) yielded an scFv with an 17.5-fold higher K<sub>d</sub>, and a much faster k<sub>off</sub> (1.38 × 10<sup>-1</sup> s<sup>-1</sup>) than wt C6.5. The remainder of the alanine substitutions yielded only minor (0.5 to 3.7-fold) increases or decreases in K<sub>d</sub>.

Based on the results of the alanine scan and a model of C6.5 based on the Fab KOL (Marquardt *et al.*, 1980), residues H95A, C100, and C100e were not mutated due to their probability of having an important structural role. H95 is likely to be buried at the V<sub>H</sub>-V<sub>L</sub> interface where it makes critical packing contacts with the V<sub>L</sub> domain. The two cysteine residues also are likely to have a structural role in maintaining the miniloop conformation. W100h was also not mutated given the unique features of tryptophan in antibody combining sites

(Mian *et al.*, 1991). The remaining 16 amino acids were completely randomized four residues at a time in four separate C6VHCDR3 libraries (96 to 99, library A; 100a to 100d, library B; 100f, 100g, 100i,

and 100j, library C, and 100k to 102, library D; see Table 4). After transformation, libraries were obtained with sizes  $1.7 \times 10^7$  (library A),  $1.3 \times 10^7$  (library B),  $3.0 \times 10^6$  (library C), and  $2.4 \times 10^7$



**Figure 1.** Model of the location of mutations in  $V_L$  CDR3. The location of mutations present in higher affinity scFv isolated from the  $V_L$  CDR3 library were modeled on the structure of the Fab fragment of the immunoglobulin KOL (Marquart *et al.*, 1980). A,  $V_L$  domain of KOL, rotated to view the four  $\beta$ -strands (yellow) that pack on the  $V_H$  domain at the  $V_H$ - $V_L$  interface.  $V_L$  CDR3 residues which are conserved in mutant scFv are shown in green (A89, A90, W91, D92, and G 95b) and those that are not conserved and modulate affinity are shown in red (D93, S94, L95, and S95a). CDR3 residues which are contributed by the joining (J) gene segment were not mutated and are shown in blue (W 96 and V 97). Framework residues comprising the four  $\beta$ -strands that pack at the  $V_H$ - $V_L$  interface are shown in yellow. Conserved residues extend the  $\beta$ -strands, while non-conserved amino acids form a four-residue loop. B, Same view as A, but with side-chains represented in space filling format. Side-chains of conserved residues (A89, A90, W91) pack directly or indirectly at the  $V_H$ - $V_L$  interface. Non-conserved residues have solvent accessible side-chains. C, View from above, looking down on the antibody combining site. The  $V_H$  domain is dark blue (right) and the  $V_L$  domain is light gray (left).  $V_H$  CDR1,  $V_H$  CDR2,  $V_L$  CDR1, and  $V_L$  CDR2 are colored magenta. Conserved  $V_L$  CDR3 residues (green) are buried, at the  $V_H$ - $V_L$  interface, and by the  $V_H$  CDR3 (dark gray) except for a portion of the side-chain of W91. Non-conserved residues (red) have solvent accessible side-chains, with D93, the most frequently mutated residue, located in the center of the binding pocket.

**Table 3.** Binding kinetics of C6.5 V<sub>H</sub> CDR3 mutants obtained by alanine scanning

sFv clone	$K_d(\text{mutant})/$ $K_d(\text{C6.5})$	$K_d$ ( $10^{-9}$ M)	$k_{on}$ ( $10^5 \text{ M}^{-1} \text{ s}^{-1}$ )	$k_{off}$ ( $10^{-2} \text{ s}^{-1}$ )
C6.5H95A	NB	NB	NB	NB
C6.5D96A	2.8	4.5	$2.2 \pm 0.34$	$1.0 \pm 0.02$
C6.5V97A	3.0	4.8	$3.1 \pm 0.62$	$1.5 \pm 0.02$
C6.5G98A	19.8	31.7	$4.1 \pm 0.71$	$13 \pm 0.55$
C6.5Y99A	3.7	5.9	$9.0 \pm 0.17$	$5.3 \pm 0.07$
C6.5C100S/C100eS	17.5	28.0	$5.0 \pm 0.25$	$13.8 \pm 0.71$
C6.5S100aA	1.8	2.8	$4.7 \pm 0.55$	$1.3 \pm 0.04$
C6.5S100bA	2.9	4.7	$3.4 \pm 0.49$	$1.6 \pm 0.07$
C6.5S100cA	1.5	2.4	$4.5 \pm 0.62$	$1.1 \pm 0.03$
C6.5N100dA	1.8	2.9	$4.1 \pm 0.34$	$1.2 \pm 0.05$
C6.5K100gA	0.6	0.98	$4.3 \pm 0.31$	$0.42 \pm 0.01$
C6.5W100hA	NB	NB	NB	NB
C6.5P100iA	0.6	1.0	$10.5 \pm 0.12$	$1.1 \pm 0.02$
C6.5E100jA	NB	NB	NB	NB
C6.5Y100kA	101.0	161.6	$0.73 \pm 0.07$	$11.8 \pm 0.25$
C6.5F100lA	28.4	45.4	$1.1 \pm 0.13$	$5.0 \pm 0.06$
C6.5Q101A	0.5	0.82	$12.0 \pm 0.02$	$0.98 \pm 0.02$
C6.5H102A	1.2	1.9	$5.9 \pm 0.57$	$1.1 \pm 0.02$

Amino acid residues 95 to 99, 100a to 100d, and 100g to 102 of C6.5 V<sub>H</sub> CDR3 were mutated to alanine using site-directed mutagenesis. Cysteine residues, C100 and C100e, were simultaneously mutated to serine.  $k_{on}$  and  $k_{off}$  were measured by SPR in a BIAcore, and the  $K_d$  calculated. Numbering is according to Kabat *et al.* (1987). NB, no binding.

(library D). The mutant phage antibody libraries were designated C6VHCDR3 libraries A, B, C, and D. PCR screening revealed that 30 of 30 randomly selected colonies from each library had full length insert and diversity was confirmed by sequencing ten unselected clones from each library (results not shown). Prior to selection, the percent of clones expressing scFv which bound c-erbB-2 ECD by ELISA was 1% for C6VHCDR3 library A, 57%, library B, 2% library C, and 3% library D. The C6VHCDR3 libraries A, B, C, and D were selected on biotinylated c-erbB-2 ECD as described above and by Schier *et al.* (1996b), but using lower antigen concentration. The first round of selection was performed using  $5.0 \times 10^{-9}$  M c-erbB-2 ECD, tenfold lower than for the first round of selection of the C6VLCDR3 library. This concentration was chosen because the parental scFv for these libraries (C6ML3-9) had a greater than tenfold lower  $K_d$  than the parental clone for the C6VLCDR3 library (C6.5). Biotinylated c-erbB-2 ECD concentration was then decreased 100-fold for the second round of selection ( $5.0 \times 10^{-11}$  M) and tenfold for the third and fourth rounds ( $5.0 \times 10^{-12}$  M and  $5.0 \times 10^{-13}$  M). As for the C6VLCDR3 library, the rate of binding of polyclonal phage was measured in a BIAcore to determine the antigen concentration used for the subsequent round of selection (Schier & Marks, 1996).

#### Characterization of mutant scFv

After four rounds of selection, positive clones were identified by ELISA and at least 24 scFv from the fourth round of selection were ranked by  $k_{off}$  using SPR in a BIAcore. The ten scFv with the lowest  $k_{off}$  from C6VHCDR3 libraries A, C, and D

were sequenced. Due to the diversity of isolated scFv in C6VHCDR3 library B, 48 scFv were ranked by  $k_{off}$  using SPR, and 22 clones with the lowest  $k_{off}$  were sequenced. scFv were purified by IMAC, followed by gel filtration to remove any dimeric or aggregated scFv. The  $k_{on}$  and  $k_{off}$  were determined by BIAcore and the  $K_d$  calculated. Very different results were obtained from the four libraries with respect to the number of higher affinity scFv isolated, and the value of the highest affinity scFv. The best results were obtained from library B (Table 4). Fifteen scFv were isolated with a  $K_d$  lower than wt C6ML3-9 and no wt sequences were observed. The best scFv (C6MH3-B47) had a  $K_d = 1.1 \times 10^{-10}$  M, ninefold lower than C6ML3-9 and 145-fold lower than C6.5. The  $k_{off}$  of this scFv was  $7.5 \times 10^{-5} \text{ s}^{-1}$ , tenfold lower than C6ML3-9 and 84-fold lower than C6.5. While a wide range of sequences was observed, a subset of scFv had the consensus sequence TDRT (first eight scFv, Table 4, library B). The consensus sequence is identical with the sequence of C6MH3-B1, which is the scFv with the lowest  $k_{off}$  ( $6.0 \times 10^{-5} \text{ s}^{-1}$ ). Five scFv were isolated that had a  $k_{off}$  2.5 to 3.75-fold lower than C6ML3-9, however expression levels were too low to obtain adequate purified scFv for measurement of the  $K_d$  (last five sequences, Table 4, library B).

The next best results were obtained from library D (Table 4, library D). Five higher affinity scFv were isolated, with the best having a  $K_d$  sevenfold higher than wt C6ML3-9. An additional scFv was isolated that had a  $k_{off}$  lower than wt scFv, however the expression level was too low to obtain adequate purified scFv for measurement of the  $K_d$  (last sequence, Table 4, library D). There was selection for a consensus mutation of Y100kW and replace-

ment of F1001 with hydrophobic methionine or leucine.

No higher affinity scFv were isolated from either the A or C libraries. From library A, 8/10 scFv were wt, with one higher affinity scFv, a contaminant from library B. A single mutant scFv with the conservative replacement of Y99F had an apparent  $k_{off}$  2.5 times lower than wt, but expression levels were too low to obtain adequate purified scFv to measure the  $K_d$ . From library C, 8/10 scFv were wt scFv, with one higher affinity scFv having mutations located in the  $V_H$  and  $V_L$  genes, but not in the region intentionally mutated. The isolated mutant scFv K100gV had a  $K_d$  2.7-fold lower than wt ( $k_{off}$  3.8-fold lower than C6ML3-9).

#### Ability of alanine scanning to identify residues which modulated affinity

Residue E100j, the only residue that when converted to alanine had no detectable binding,

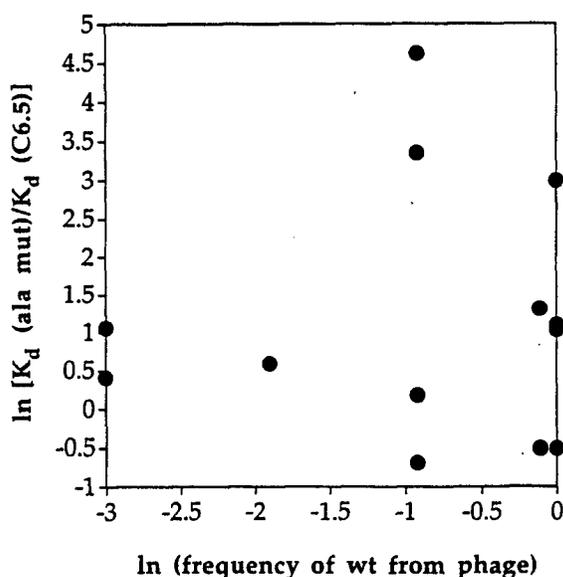
was 100% conserved. Otherwise, there was no correlation between the frequency with which the wt amino acid was recovered and the extent to which binding was reduced by substitution to alanine (Figure 2). Similarly, there was no correlation between residues shown to modulate affinity by alanine scanning and mutations exhibiting improved binding. This is clear when comparing the results obtained from library B (where no alanine mutant had more than a 2.9-fold increase in  $K_d$ ) and library D (where  $K_d$  was markedly increased for two alanine mutants, Y100kA and F1001A). Despite the different alanine scan results, both libraries yielded similar nine and sevenfold increases in affinity. This result appears to be different than the results of Lowman & Wells (1993) who found a mild ( $R^2 = 0.71$ ) positive correlation between the frequency with which the wt amino acid was recovered from a phage library of human growth hormone mutants and the extent to which binding was reduced by alanine scanning. In

Table 4. Sequences, affinities and binding kinetics of scFv isolated from heavy chain CDR3 libraries A, B, C, and D

Clone	F	$V_H$ CDR3 sequence	Other mutations	$K_d$ ( $10^{-10}$ M)	$k_{on}$ ( $10^5$ M $^{-1}$ s $^{-1}$ )	$k_{off}$ ( $10^{-4}$ s $^{-1}$ )
C6.5	—	HDVGYCSSSNCAKWPEYFQH		160.0	4.0 ± 0.20	63.0 ± 0.60
$V_H$ CDR3 library A						
C6ML3-9 (wt)	8	-DVG Y-----		10.0	7.6 ± 0.20	7.6 ± 0.30
C6MH3-A2	1	----F-----		nd	nd	2.9 ± 0.03 <sup>a</sup>
C6MH3-A3	1	-----D-----		2.5	9.9 ± 0.52	2.5 ± 0.47
$V_H$ CDR3 library B						
C6ML3-9 (wt)	0	-----SSSN-----		10.0	7.6 ± 0.20	7.6 ± 0.30
C6MH3-B47	1	-----TDRS-----		1.1	6.7 ± 0.63	0.75 ± 0.04
C6MH3-B1	1	-----TDRT-----		1.2	5.0 ± 0.24	0.60 ± 0.06
C6MH3-B39	1	-----TDPT-----		1.8	10.7 ± 0.84	1.9 ± 0.29
C6MH3-B48	1	-----TDPS-----		2.3	5.6 ± 0.35	1.3 ± 0.01
C6MH3-B11	1	-----DRS-----		3.0	7.7 ± 0.41	2.3 ± 0.08
C6MH3-B5	1	-----TDAT-----		3.4	6.8 ± 0.39	2.3 ± 0.07
C6MH3-B41	1	-----TDRP-----		5.3	5.1 ± 0.27	2.7 ± 0.02
C6MH3-B2	1	-----TDPR-----		5.8	5.5 ± 0.38	3.2 ± 0.08
C6MH3-B20	1	-----PAR-----		1.4	11.3 ± 1.29	1.6 ± 0.36
C6MH3-B16	1	-----ADV R-----		2.0	8.0 ± 0.48	1.6 ± 0.40
C6MH3-B25	2	-----LTTR-----		2.3	8.3 ± 0.54	1.9 ± 0.32
C6MH3-B21	1	-----TTPL-----		2.6	9.1 ± 0.57	2.4 ± 0.23
C6MH3-B27	1	-----KN-R-----		4.7	8.5 ± 0.36	4.0 ± 0.47
C6MH3-B9	2	-----KTAA-----		4.6	7.2 ± 0.36	3.3 ± 0.43
C6MH3-B15	1	-----E-R-----		5.9	5.1 ± 0.49	3.0 ± 0.06
C6MH3-B34	1	-----QTDR-----	VL Q1R	nd	nd	2.0 ± 0.04 <sup>a</sup>
C6MH3-B43	1	-----EDYT-----	VH K23Q	nd	nd	2.6 ± 0.04 <sup>a</sup>
C6MH3-B46	1	-----TTPR-----	VH V76G	nd	nd	2.8 ± 0.03 <sup>a</sup>
C6MH3-B33	1	-----DQT-----		nd	nd	2.8 ± 0.04 <sup>a</sup>
C6MH3-B31	1	-----DDYT-----	VL P7L	nd	nd	2.9 ± 0.04 <sup>a</sup>
$V_H$ CDR3 library C						
C6ML3-9 (wt)	8	-----AKWPE-----		10.0	7.6 ± 0.20	7.6 ± 0.30
C6MH3-C4	1	-----V-----		3.7	5.4 ± 0.94	2.0 ± 0.21
C6MH3-C3	1	-----	VH G15E	6.5	4.9 ± 0.57	3.2 ± 0.01
$V_H$ CDR3 library D						
C6ML3-9 (wt)	4	-----YFQH-----	VL N54D	10.0	7.6 ± 0.20	7.6 ± 0.30
C6MH3-D2	1	-----WLG V-----		1.4	8.3 ± 0.38	1.2 ± 0.02
C6MH3-D3	1	-----WLDN-----		2.7	7.4 ± 0.35	2.0 ± 0.25
C6MH3-D6	1	-----WMYP-----		3.5	5.2 ± 0.18	1.8 ± 0.01
C6MH3-D5	1	-----WM-M-----		5.8	3.6 ± 0.21	2.1 ± 0.02
C6MH3-D1	1	-----WLHV-----		7.5	3.6 ± 0.04	2.7 ± 0.04
C6MH3-D7	1	-----WQDP-----	VL N54S	nd	nd	3.1 ± 0.09 <sup>a</sup>

$k_{on}$  and  $k_{off}$  were determined in a BiAcCore using purified scFv, and  $K_d$  calculated. Dashes indicate sequence identity. Mutations arising from PCR error and located outside  $V_H$  CDR3 are listed under the heading other mutations. F, frequency of isolated scFv.

<sup>a</sup>  $k_{off}$  determined from unpurified scFv samples.

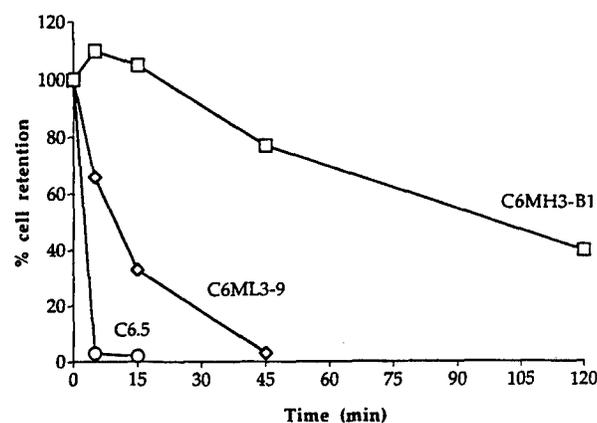


**Figure 2.** Relationship between the frequency with which a wild-type amino acid is recovered from  $V_H$  CDR3 phage antibody libraries and the extent to which binding is reduced when the residue is converted to alanine. The relative change in binding affinity from wt for alanine substitutions is plotted as  $\ln [K_d (\text{Ala mutant})/K_d (\text{C6.5})]$ . Data for residue N100d could not be used because the wt residue was not recovered at this position. Data for residue E100j could not be used because no binding could be detected. No correlation exists between the frequency with which a wt amino acid is recovered from  $V_H$  CDR3 phage antibody libraries and the extent to which binding is reduced when the residue is converted to alanine.

addition, their largest improvements in affinity were for those residues shown by alanine scanning to significantly affect binding. The reason for the different results is unclear, however in two of our  $V_H$  CDR3 libraries where alanine scanning indicated a significant effect on binding (library A and C), expression levels of mutants were generally low. This could have affected the selection results.

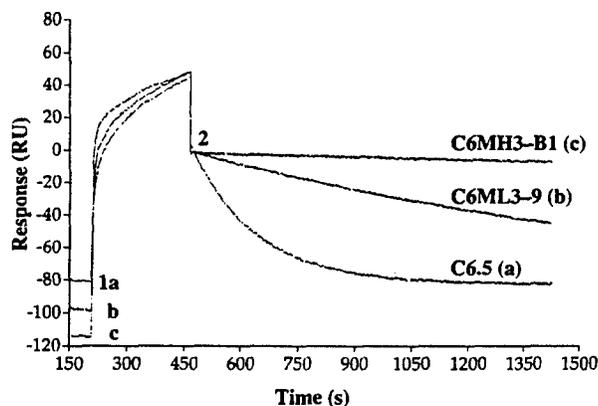
#### Correlation between affinity and cell surface retention of scFv

The retention of biotinylated C6.5, C6ML3-9, and C6MH3-B1 scFv on the surface of SK-OV-3 cells expressing c-erbB-2 was determined, both to verify the observed differences in  $k_{off}$ , and to confirm that the antigen as presented in the BIAcore had biologic significance. The half life ( $t_{1/2}$ ) of the scFv on the cell surface was much less than five minutes for C6.5, 11 minutes for C6ML3-9, and 102 minutes for C6MH3-B1 (Figure 3). These values agree closely with the  $t_{1/2}$  calculated from the  $k_{off}$  as determined by SPR in a BIAcore (1.6 minutes for C6.5, 13 minutes for C6ML3-9, and 135 minutes for C6MH3-B1; Figure 4). The anti-digoxin scFv 26-10 (Huston *et al.*, 1988) was used as



**Figure 3.** Cell surface retention of wild-type and mutant scFv. Retention of biotinylated C6.5 ( $k_{off} = 6.3 \times 10^{-3} \text{ s}^{-1}$ ) on the surface of c-erbB-2 expressing SK-OV-3 cells was determined by FACS and compared to the retention of C6ML3-9 ( $k_{off} = 7.6 \times 10^{-4} \text{ s}^{-1}$ ) and C6MH3-B1 ( $k_{off} = 6.0 \times 10^{-5} \text{ s}^{-1}$ ). The half life ( $t_{1/2}$ ) of the scFv on the cell surface was much less than five minutes for C6.5, 11 minutes for C6ML3-9, and 102 minutes for C6MH3-B1. These values agree closely with the  $t_{1/2}$  calculated from the  $k_{off}$  as determined by SPR in a BIAcore (1.6 minutes for C6.5, 13 minutes for C6ML3-9, and 135 minutes for C6MH3-B1; Figure 3).

negative control, and no binding to c-erbB-2 ECD in a BIAcore or to c-erbB-2 on SK-OV-3 cells was observed.



**Figure 4.** Dissociation of wild-type and mutant scFv from a c-erbB-2 ECD coupled sensor chip in a BIAcore. Overlay plot of sensorgrams of the association and dissociation of C6.5, C6ML3-9, and C6MH3-B1 scFv to a c-erbB-2 coupled sensor chip in a BIAcore. The decrease in  $k_{off}$  which accounts for the majority of the increase in  $K_d$  correlates closely with the  $t_{1/2}$  of retention on SK-OV-3 cells (Figure 2). Sensorgrams were positioned so that the amount (RU) of scFv bound at the beginning of dissociation was superimposed. For this figure,  $5.0 \times 10^{-7} \text{ M}$  scFv were injected over a BIAcore flow cell coated with 1000 RU of c-erbB-2 ECD under a constant flow of 25  $\mu\text{l}/\text{minute}$ . scFv were allowed to dissociate for a time period of 15 minutes. (1) Injection of scFv and beginning of the association phase. (2) End of the association phase, beginning of the dissociation phase. a, C6.5; b, C6ML3-9; c, C6MH3-B1.

**Table 5.** Binding kinetics of scFv derived from C6.5 V<sub>L</sub> CDR3, V<sub>H</sub> CDR3 and light chain shuffled mutants

Clone	K <sub>d</sub> (10 <sup>-10</sup> M)	k <sub>on</sub> (10 <sup>5</sup> s <sup>-1</sup> M <sup>-1</sup> )	k <sub>off</sub> (10 <sup>-4</sup> s <sup>-1</sup> )	K <sub>d</sub> (parent) K <sub>d</sub> (mut)	K <sub>d</sub> (C6.5) K <sub>d</sub> (mut)	ΔΔG <sub>i</sub> (kcal/mol)
<b>A. Combined mutants: C6ML3-9 or C6ML3-12 with light chain shuffled C6L1</b>						
C6-9L1	3.3	9.2 ± 0.20	3.0 ± 0.40	3.0	49	+0.42
C6-12L1	1.9	6.7 ± 0.12	1.3 ± 0.32	8.4	84	-0.18
<b>B. Combined mutants: C6MH3-B1 or C6MH3-B47 with light chain shuffled C6L1</b>						
C6-B1L1	6.3	3.8 ± 0.19	2.4 ± 0.01	0.19	25	+0.43
C6-B47L1	6.0	3.0 ± 0.16	1.8 ± 0.01	0.18	27	+0.45
<b>C. Combined mutants: C6MH3-B1 or C6MH3-B47 with D library mutants</b>						
C6-B1D1	0.32	4.7 ± 0.31	0.15 ± 0.005	3.8	500	-0.61
C6-B1D2	0.15	6.9 ± 0.42	0.10 ± 0.014	8.0	1067	-0.07
C6-B1D3	0.13	6.4 ± 0.20	0.08 ± 0.002	9.2	1231	-0.53
C6-B1D5	0.35	5.1 ± 0.36	0.18 ± 0.001	3.4	457	-0.40
C6-B1D6	0.32	4.1 ± 0.17	0.13 ± 0.002	3.8	500	-0.16
C6-B47D1	0.68	7.1 ± 0.95	0.48 ± 0.001	1.6	235	-0.11
C6-B47D2	0.44	9.8 ± 0.72	0.43 ± 0.001	2.5	364	+0.62
C6-B47D3	0.48	6.6 ± 0.26	0.32 ± 0.001	2.3	333	+0.29
C6-B47D5	0.63	6.2 ± 0.31	0.39 ± 0.002	1.7	254	-0.01
C6-B47D6	0.51	5.9 ± 0.30	0.30 ± 0.001	2.2	314	+0.17

A, Mutants produced by combining the V<sub>L</sub> CDR3 of C6ML3-9 or C6ML3-12 with the V<sub>L</sub> gene of the C6.5 light chain shuffled scFv, C6L1 (Schier *et al.*, 1996b). B, Mutants produced by introducing mutations in FR1 to FR3 of C6L1 light chain into C6MH3-B1 or C6MH3-B47. C, Mutants obtained by combining mutations of C6MH3-B1 or C6MH3-B47 with mutations from D library clones (D1, D2, D3, D5, D6). k<sub>on</sub>, and k<sub>off</sub> were measured by SPR in a BIAcore, and the K<sub>d</sub> calculated. ΔΔG<sub>i</sub> were calculated as by Lowman & Wells (1993), and Cunningham & Wells (1993).

### Effects on binding kinetics by combining mutations from high affinity scFv

To further increase affinity, the sequences of the two highest affinity scFv obtained from the V<sub>H</sub> CDR3B library (C6MH3-B1 or C6MH3-B47) were combined with the sequences of scFv isolated from the C6VHCDR3D library (C6MH3-D1, -D2, -D3, -D5, or -D6). An increase in affinity from wt was obtained for all these combinations, yielding an scFv (C6-B1D3) that had a 1230-fold lower K<sub>d</sub> than wt C6.5 (Table 5). The extent of additivity varied considerably, however, and could not be predicted from the parental k<sub>on</sub>, k<sub>off</sub>, or K<sub>d</sub>. In some combinations, cooperativity was observed, with a negative ΔΔG<sub>i</sub>. Additional combinations were made between a previously described light chain shuffled C6.5 mutant (C611, sixfold decreased K<sub>d</sub>; Schier *et al.*, 1996b) and one of two V<sub>L</sub> CDR3 mutants (C6ML3-9 and C6ML3-12). These combinations yielded scFv with 49 and 84-fold improved affinity (Table 5). Introducing the same rearranged V<sub>L</sub> gene into the highest affinity V<sub>H</sub> CDR3 mutants (C6MH3-B1 or C6MH3-B47) resulted in decreased affinity compared to C6MH3-B1 (Table 5). A similar effect was described in previous work (Schier *et al.*, 1996b) when rearranged V<sub>L</sub> and V<sub>H</sub> genes from high affinity chain shuffled scFv obtained from parallel selection were combined.

### Discussion

Ultra-high affinity scFv were engineered by diversifying the CDRs that comprise the center of the antibody combining site. Sequential diversification of V<sub>L</sub> and V<sub>H</sub> CDR3 yielded scFv with up to a

145-fold increase in affinity (K<sub>d</sub> = 1.1 × 10<sup>-10</sup> M). Combination of these mutations with independently selected mutations located elsewhere in V<sub>H</sub> CDR3 yielded an additional ninefold increase in affinity (K<sub>d</sub> = 1.3 × 10<sup>-11</sup> M). The scFv were produced without any immunization and have higher affinity than any antibody fragments engineered *in vitro*. The results illustrate the power of diversity libraries and phage display to produce antibody fragments with affinities rarely achieved by immunization (Foote & Eisen, 1995) and have important implications for the design of mutant phage antibody libraries. Moreover, the availability of such high affinity antibody fragments may have important consequences for antibody based tumor targeting.

### Accuracy of affinity measurements

The validity of our results depends on the accuracy of the measured affinities, which were calculated from k<sub>on</sub> and k<sub>off</sub> determined by BIAcore. To verify that differences in k<sub>on</sub> were not due to differences in the immunoreactivity of the purified scFv, the concentration of functional scFv was determined by measuring the binding rate to c-erbB-2 ECD under mass transport limited conditions (Karlsson *et al.*, 1993; Schier *et al.*, 1996b). Since increases in affinity were largely due to a decrease in k<sub>off</sub>, precautions were taken to avoid the introduction of artifact into these measurements (Nieba *et al.*, 1996). Purified scFv were gel filtered immediately prior to k<sub>off</sub> measurement, to avoid avidity from dimeric or aggregated antibody fragment (Schier *et al.*, 1996b), and analytical gel filtration was performed after measurement of k<sub>off</sub>

to confirm the absence of aggregated material. To minimize the probability of rebinding,  $k_{\text{off}}$  was measured using a high flow rate and a scFv concentration that resulted in near saturation of the chip surface. The amount of c-erbB-2 ECD coupled to the chip surface was the lowest amount that gave an adequate binding response (100 to 150 RU) for accurate kinetic measurement. In our experience, using the minimal amount of coupled antigen is the single most important parameter for preventing rebinding. Using these experimental conditions, we were unable to detect any evidence of rebinding when  $k_{\text{off}}$  was measured in the presence of  $5 \times 10^{-7}$  M c-erbB-2 ECD in the running buffer. Furthermore, the affinity of C6.5 previously determined by Scatchard after radiolabeling ( $2.0 \times 10^{-8}$  M, Schier *et al.*, 1995) agrees closely to the value determined by BIAcore ( $1.6 \times 10^{-8}$  M). The  $k_{\text{off}}$  of CDR3 mutants determined by cell surface retention of biotinylated scFv also agrees closely to  $k_{\text{off}}$  measured by BIAcore. Engineering further increases in affinity is likely to require a different technique for affinity measurement, since the binding kinetics of the highest affinity scFv are near the limit of measurement using SPR in a BIAcore ( $k_{\text{on}} > 10^6 \text{ M}^{-1} \text{ s}^{-1}$  and  $k_{\text{off}} < 10^{-5} \text{ s}^{-1}$ ; Malmqvist, 1993). Determining  $k_{\text{off}}$  below  $10^{-5} \text{ s}^{-1}$  is difficult due to the small amount of analyte dissociating (1% in 17 minutes), the background noise, and disturbances from the pumps and valves of the flow system. Determination of  $K_d$  in the BIAcore using competition experiments (Nieba *et al.*, 1996) will also be limited by instrument sensitivity.

#### Design of mutant antibody libraries

When designing a mutant phage antibody library, decisions must be made as to how and where to introduce mutations. Mutations can be randomly introduced, using either chain shuffling (Clackson *et al.*, 1991; Marks *et al.*, 1992), error prone PCR (Hawkins *et al.*, 1992), or mutator strains (Low *et al.*, 1996), thus apparently mimicking the process of somatic hypermutation. These approaches have yielded large increases in affinity for hapten antigens (>100-fold; Low *et al.*, 1996; Marks *et al.*, 1992), but results with protein binding antibody fragments have been more modest (<tenfold) (Hawkins *et al.*, 1992; Schier *et al.*, 1996b). Moreover, the relatively random distribution of mutations in higher affinity clones provides little useful information as to where to direct additional mutations. Alternatively, knowledge of the general structure of the Fv fragment and its complexes with antigen can be used to direct mutagenesis to the CDRs that form the contact interface between antibody and antigen.

Targeting mutations to the CDRs has previously been shown to be an effective technique for increasing antibody affinity. Yang *et al.* (1995) increased the affinity of an anti-HIV gp120 Fab 420-fold ( $K_d = 1.5 \times 10^{-11}$  M) by mutating four CDRs in five libraries and combining independently

selected mutations. We achieved three times that increase in affinity by mutating a much smaller portion of the antibody combining site contained within only two CDRs. Our results may be partly due to the stringent selection conditions used and the techniques used to monitor selections and screen for higher affinity scFv without the need for purification. However, the results also suggest that focusing mutations in  $V_H$  and  $V_L$  CDR3 may be a more efficient means to increase affinity.

Directing mutations into  $V_H$  and  $V_L$  CDR3 to increase affinity may initially seem at odds with studies on antibody structure and function. Although 15 to 22 amino acids located in loops within the CDRs typically contact antigen (Davies *et al.*, 1990), free energy calculations and mutational analysis indicate that only a small subset of the contact residues contribute the majority of the binding energy (Hawkins *et al.*, 1993; Kelley & O'Connell, 1993; Novotny *et al.*, 1989). The high energy contact residues are more frequently located in the center of the antibody combining sites in the  $V_H$  and  $V_L$  CDR3s. Thus, mutation of  $V_H$  and  $V_L$  CDR3 is more likely to destroy high affinity contacts than mutation of other CDRs. However, these residues will be recreated, albeit at low frequency, given an adequate library size for the number of residues randomized. Mutant residues could increase affinity by introducing new contact residues (Alzari *et al.*, 1990) or by replacing low affinity (Novotny *et al.*, 1989; Kelley & O'Connell, 1993) or "repulsive" contact residues (Novotny *et al.*, 1989) with contact residues with more favorable energetics. It appears, however, that many mutations introduced either by somatic hypermutation *in vivo* (Sharon, 1990) or mutagenesis *in vitro* (Hawkins *et al.*, 1993) exert their effect on affinity indirectly, in many instances by precisely positioning the side-chains of contact residues for optimal electrostatic, hydrogen bonding, and van der Waals interactions (Mian *et al.*, 1991). Mutation of non-contact CDR residues located close to high energy contact CDR residues may be more likely to exert this indirect effect. The importance of the CDR3s as sites for mutagenesis is also supported by the work of Yang *et al.* (1995) who created separate libraries of  $V_L$  CDR1,  $V_L$  CDR3,  $V_H$  CDR1, and  $V_H$  CDR3 mutants. The largest increases in affinity were 7.9 and 7.7-fold from sequential mutation of two separate regions of  $V_H$  CDR3, resulting in a 63-fold increase in affinity over wt Fab. Mutation of  $V_L$  CDR3 resulted in the next largest increase in affinity over wt (5.6-fold).

Directing mutations into  $V_H$  and  $V_L$  CDR3 to increase affinity may also appear to be different than the locations where mutations are directed and accumulate during somatic hypermutation *in vivo*. Germline diversity is greatest in the center of the antibody combining site (Tomlinson *et al.*, 1996), particularly  $V_H$  and  $V_L$  CDR3, where tremendous sequence diversity is generated by recombination, N segment addition, and joining diversity. Somatic hypermutation extends sequence

diversity to CDR residues located more peripherally in the antibody combining site (Tomlinson *et al.*, 1996). On closer inspection, however, striking similarities exist between our results and somatic hypermutation *in vivo*. Nucleotide substitutions *in vivo* are not targeted randomly, but rather occur at specific sequence hotspots intrinsic to the mutational process, for example at serine residues encoded by the nucleotides AGY but not at serine residues encoded by TCN (Betz *et al.*, 1993; Reynaud *et al.*, 1995). Serine residues encoded by AGY have previously been shown to predominate over those encoded by TCN in CDR1 and two of the  $V_H$  domain and CDR1 of the  $V_K$  domain, but not in the frameworks (Betz *et al.*, 1993; Wagner *et al.*, 1995). The germline gene segments encoding the  $V_K$  and  $V_L$  CDR3s are similarly biased to contain a high proportion of AGY serine (Figure 5). The CDR3 AGY/TCN ratio is 9.7 for the  $V_K$  gene segments and 5.4 for the  $V_L$  gene segments. These values are comparable to the AGY/TCN ratios observed for  $V_H$  CDR1 (20.3),  $V_H$  CDR2 (2.28), and  $V_K$  CDR1 (8.75) and are greater than values observed for framework residues (0.3 to 0.67; Wagner *et al.*, 1995). Thus the sequences encoding the  $V_L$  CDR3 have evolved to be targets of the somatic hypermutation machinery. Accordingly, an extensive analysis of the location of mutations introduced by somatic hypermutation into the germline  $V_K$  genes identified CDR3 as a frequent site of mutagenesis (Tomlinson *et al.*, 1996). Within  $V_K$  CDR3, residues 89 to 91 are most conserved, with the highest frequency of mutation observed at residue 93 (Tomlinson *et al.*, 1996). Similarly, during *in vitro* affinity maturation of the C6.5  $V_L$  CDR3, we observed conservation of residues 89 to 92, and 95b (this work) and 96 (Schier *et al.*, 1996a). In contrast, substitution occurred at residues 93 to 95a, with the highest frequency of mutation at residue 93. The location of mutations parallels exactly the distribution of AGY serine residues within the germline  $V_L$  CDR3 genes (Figure 5).

Such a detailed analysis is more difficult for  $V_H$  CDR3 since germline D gene segment assignment is frequently not possible, and the  $V_H$  CDR3 loop cannot be accurately modeled. In 33 published D gene segments (Kabat *et al.*, 1991), the ratio of AGY/TCN serine residues is 2.5/1, a value closer to that observed for CDRs (2.3 to 20.3) than for framework residues (0.3 to 0.67). Thus portions of the  $V_H$  CDR3 also appear to be targets for somatic hypermutation *in vivo*. In the case of the C6.5, the sequence motif CSSSNC located within the  $V_H$  CDR3 is encoded by the germline D gene segment D1 (Kabat *et al.*, 1991) which encodes the sequence CSSTSC. In the germline gene, all three serine residues are encoded by AGY and in C6.5 these three residues were hotspots for substitutions which increased affinity. Moreover, an extremely wide range of amino acid residues were tolerated between the two cysteine residues, as evidenced by the alanine scan results and the fact that 57% of unselected scFv bound antigen. In contrast, only 1

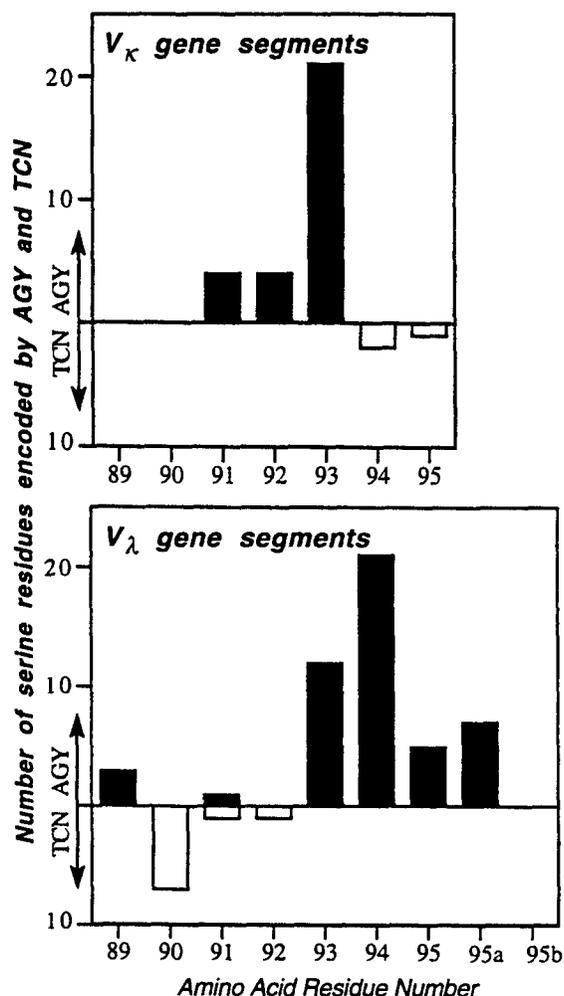


Figure 5. Frequency of serines encoded by AGY or TCN in the CDR3s of the  $V_K$  and  $V_L$  germline gene segments. The DNA sequences of 50 germline  $V_K$  genes (Cox *et al.*, 1994) and 24 germline  $V_L$  genes (Williams & Winter, 1993) were analyzed and the serine codon usage (AGY or TCN) plotted as a function of residue number within the CDR3 (residues 89 to 95 for  $V_K$  and 89 to 95b for  $V_L$ ). The number of serine residues encoded by AGY are shown by black bars and the number of serine residues encoded by TCN by open bars.

to 2% of unselected scFv from the three other  $V_H$  CDR3 libraries bound antigen. One possible explanation is that the disulfide bond formed by the cysteine residues stabilizes this region of the  $V_H$  CDR3. Regardless, five of the 33 D gene segments encode a CXXXXC or CXXXC (Kabat *et al.*, 1991) in the preferred reading frame (Yamada *et al.*, 1991; Abergel & Claverie, 1991) suggesting that the motif has useful properties as a component of the primary immune repertoire. If so, then use of similar motifs could prove useful in construction of semi-synthetic antibody libraries (Hoogenboom & Winter, 1992).

Modeling the location of the observed mutations in  $V_L$  CDR3 on the Fab structure KOL, where the  $V_L$  is derived from the same germline gene as C6.5,

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suggested a basis for the pattern of conservation and substitution we observed. The conserved residues appear to have a significant structural role in the variable domain, either in maintaining the main-chain conformation of the loop, or in packing on the  $V_H$  domain. In our model, the side-chain of W91 is buried at the  $V_H$ - $V_L$  interface and by the long  $V_H$  CDR3 of KOL. In other structures, the side-chain of  $V_L$ 91 is at least partially solvent accessible, and frequently contacts antigen (Mian *et al.*, 1991; Tomlinson *et al.*, 1996). In contrast to the extensive packing of the side-chains of conserved residues, all but one of the four most extensively substituted residues appear to have solvent accessible side-chains. A similar pattern was also observed during parsimonious mutagenesis of C6.5  $V_L$  CDR1 and  $V_H$  CDR2 (Schier *et al.*, 1996a). Residues within the CDRs with structural roles were conserved, while substitutions were largely confined to residues with solvent accessible side-chains. Analysis of the location of substitutions in  $V_H$  and  $V_L$  genes mutated *in vivo* also indicates that residues involved in maintaining the main-chain conformation rarely undergo non-conservative substitution (Chothia *et al.*, 1992; Tomlinson *et al.*, 1995).

The previous analysis suggests a mutagenesis strategy for efficiently increasing antibody fragment affinity. Mutagenesis is directed into  $V_L$  and  $V_H$  CDR3 sequentially, as in this work, rather than by parallel evolution of the two CDRs. These two CDRs pack on each other, and mutations isolated in parallel are likely to not be additive (Yang *et al.*, 1995). Mutagenesis is initially directed into  $V_L$  CDR3 due to the limited main-chain conformations (Chothia & Lesk, 1987; Tomlinson *et al.*, 1995) and the ability to model the CDR on a homologous Fv or Fab structure. Modeling should be used to identify CDR residues that are likely to have a structural role, either in maintaining the main-chain conformation or in packing against the  $V_H$  domain. These residues are conserved, leaving at most four to five residues which can be completely randomized in a reasonably sized library ( $10^7$  to  $10^8$  member). The highest affinity  $V_L$  CDR3 mutant is then used as a template for  $V_H$  CDR3 mutagenesis. Given the length of  $V_H$  CDR3s, it is likely that it will not be possible to sample the entire sequence space simultaneously. Instead residues are randomized four to five at a time, as in this work, and independently selected mutations combined. In this and previous work (Schier *et al.*, 1996a), we observed complete conservation of the four glycine and two tryptophan residues randomized. In the CDRs, glycine residues are typically key residues in turns, and the chemical properties of tryptophan make it a frequent structural or high energy contact residue (Mian *et al.*, 1991). Thus conservation of these two residues when randomizing  $V_H$  CDR3 should be considered, if sequence space is limiting. Since combination of independently selected mutations may not be additive, it may be more prudent to simultaneously scan all residues at a low

mutation frequency (parsimonious mutagenesis) to identify residues that modulate affinity, and structural and functional residues that are conserved (Schier *et al.*, 1996a). Residues identified as modulating affinity would then be completely randomized in a second library. Alanine scanning appeared to be useful only to identify essential contact and structural residues, but not for predicting which residues would yield higher affinity when mutated. If necessary, affinity could be increased further by mutating the other CDRs. Particularly suitable might be  $V_H$  CDR1 and  $V_L$  CDR1. These CDRs appear to be more important in modulating affinity during *in vivo* affinity maturation, based both on the higher frequency of AGY serine in the germline genes (Wagner *et al.*, 1995), and a higher frequency of mutation in rearranged genes (Tomlinson *et al.*, 1996). Modeling should be performed to identify structural residues to be conserved, and residues with solvent accessible side-chains, which would be mutated.

#### Implications for antibody based tumor targeting

The availability of scFv with a range of affinities for a tumor antigen makes it possible to determine the effect of affinity on specific tumor retention. While it might appear obvious that retention should increase with increasing affinity, it has been proposed that a barrier effect exists, such that the higher affinity antibodies are trapped at the tumor edge (Fujimori *et al.*, 1990). We have examined the effect of C6.5 mutants with affinities ranging between  $3.2 \times 10^{-7}$  M (C6G98A) and  $1.0 \times 10^{-9}$  M (C6ML3-9) in *scid* mice bearing human SK-OV-3 tumors. The percent injected dose/gram of tumor at 24 hours increased from 0.19% for C6G98A to 1.42% for C6ML3-9 and the tumor:blood ratios increased from 2.6 to 17.2 (G. P. Adams *et al.*, unpublished). Thus within the range of affinities studied, there was a significant increase in tumor retention. The magnitude of the 24 hour retention, however, is significantly less than the values observed for IgG (30% ID/g) in similar models. Although an affinity of  $1.0 \times 10^{-9}$  M is considered high, the  $k_{off}$  gives a predicted  $t_{1/2}$  on the cell surface of only 13 minutes, much faster than the  $\beta$  elimination  $t_{1/2}$  of the scFv from the mouse (2.5 hours). Thus tumor retention is largely dependent on the rate of clearance from the blood. The higher affinity scFv described in this paper have a  $k_{off}$  which provide a predicted  $t_{1/2}$  on the cell surface of 24 hours, significantly longer than the clearance rate from blood. These very high affinity antibody fragments, with binding kinetics not previously available, offer the possibility of significantly greater quantitative tumor retention. The scFv could also be used as building blocks to create dimeric scFv, with yet higher apparent affinity due to avidity, and even greater tumor retention (Adams *et al.*, 1993).

## Materials and Methods

### Construction of phage antibody libraries

Mutant scFv phage antibody libraries were constructed based on the sequence of C6.5, a human scFv isolated from a non-immune phage antibody library which binds to the tumor antigen c-erbB-2 with a  $K_d = 1.6 \times 10^{-8}$  M (Schier *et al.*, 1995). For construction of a library containing  $V_L$  CDR3 mutants, an oligonucleotide ( $V_L1$ ; Table 1) was designed which partially randomized nine amino acid residues located in  $V_L$  CDR3 (Table 2). For the nine amino acids randomized, the ratio of nucleotides was chosen so that the frequency of wt amino acid was 49%. To create the library, C6.5 scFv DNA (10 ng) was amplified by PCR in 50  $\mu$ l reactions containing 25 pmol LMB3 (Marks *et al.*, 1991), 25 pmol  $V_L1$ , 250  $\mu$ M dNTPs, 1.5 mM  $MgCl_2$ , and 1  $\mu$ l (five units) *Taq* DNA polymerase (Promega) in the manufacturers buffer. The reaction mixture was subjected to 30 cycles of amplification (94°C for 30 seconds, 50°C for 30 seconds and 72°C for one minute) using a Hybaid OmniGene cyler. To introduce a *NotI* restriction site at the 3' end of the scFv gene repertoire, the PCR fragment (850 bp) was gel purified and reamplified using the primers LMB3 and  $V_L2$  (Table 1). The PCR product was purified, digested with *SfiI* and *NotI*, and ligated into pCANTAB5E (Pharmacia) digested with *SfiI* and *NotI*. Ligation mixtures were purified as previously described (Schier *et al.*, 1996b) and aliquots electroporated (Dower *et al.*, 1988) into 50  $\mu$ l *E. coli* TG1 (Gibson, 1984). Cells were grown in 1 ml SOC (Sambrook *et al.*, 1990) for 30 minutes and then plated on TYE (Miller, 1972) media containing 100  $\mu$ g ampicillin/ml and 1% (w/v) glucose (TYE-AMP-Glu). Colonies were scraped off the plates into 5 ml of 2  $\times$  TY broth (Miller, 1972) containing 100  $\mu$ g ampicillin/ml, 1% (w/v) glucose (2  $\times$  TY-AMP-Glu) and 15% (v/v) glycerol for storage at -70°C. The cloning efficiency and diversity of libraries was determined by PCR screening (Gussow & Clackson, 1989) exactly as described by Marks *et al.* (1991) and by DNA sequencing (Sanger *et al.*, 1977). The mutant phage antibody library was designated C6VLCDR3.

Four libraries of  $V_H$  CDR3 mutants were constructed. For construction of each  $V_H$  CDR3 library, oligonucleotides (VHA, VHB, VHC, and VHD; Table 1) were designed which completely randomized four amino acid residues located in  $V_H$  CDR3 (amino acid residues 96 to 99, library A; residues 100a to 100d, library B; residues 100f, 100g, 100i, and 100j, library C; and residues 100k to H102, library D; Table 3). To create the libraries, DNA encoding the  $V_H$  gene of C6.5 scFv DNA (10 ng) was amplified by PCR in 50  $\mu$ l reactions containing 25 pmol LMB3 (Marks *et al.*, 1991) and 25 pmol of either VHA, VHB, VHC, or VHD exactly as described above. The resulting PCR fragments were designated VHA1, VHB1, VHC1, and VHD1, based on the mutagenic oligonucleotide used for amplification. In four separate PCR reactions, DNA encoding the light chain, scFv linker,  $V_H$  framework 4 (FR4), and a portion of  $V_H$  CDR3 of C6ML3-9 was amplified by PCR as described above using the primers C6hisnot and either RVHA, RVHB, RVHC, or RVHD (Table 1). These amplifications yielded PCR fragments VHA2, VHB2, VHC2, and VHD2. The 5' ends of primers RVHA, RVHB, RVHC, and RVHD were designed to be complementary to the 5' ends of primers VHA, VHB, VHC, and VHD, respectively. This complementarity permits joining of the VH1 and VH2 PCR fragments

together to create a full length scFv gene repertoire using splicing by overlap extension. To create the mutant scFv gene repertoires, 200 ng of each PCR fragment (VHA1 and VHA2, VHB1 and VHB2, VHC1 and VHC2, or VHD1 and VHD2) were combined in 50  $\mu$ l PCR reaction mixtures (as described above) and cycled seven times to join the fragments (94°C for 30 seconds, 60°C for five seconds, 40°C for five seconds (RAMP: five seconds), 72°C for one minute). After seven cycles, outer primers LLMB3 and C6hisnot were added and the mixtures amplified for 30 cycles (94°C for 30 seconds, 50°C for 30 seconds, 72°C for one minute). The PCR products were purified as described above, digested with *SfiI* and *NotI*, and separately ligated into pCANTAB5E (Pharmacia) digested with *SfiI* and *NotI*. The four ligation mixtures were purified as described above and electroporated into 50  $\mu$ l *E. coli* TG1. Transformed cells were grown and plated, and libraries characterized and stored, as described above. The mutant phage antibody libraries were designated C6VHCDR3A, C6VHCDR3B, C6VHCDR3C, and C6VHCDR3D.

### Preparation of phage and selection of phage antibody libraries

Preparation of phage for selection was performed exactly as described by Schier *et al.* (1996b). Phage particles were purified and concentrated by two PEG precipitations (Sambrook *et al.*, 1990), resuspended in 5 ml phosphate buffered saline (25 mM  $NaH_2PO_4$ , 125 mM NaCl, pH 7.0, PBS) and filtered through a 0.45  $\mu$ m filter. All libraries were selected using biotinylated c-erbB-2 ECD and streptavidin-coated paramagnetic beads M280 (Dyna) as described by Schier *et al.* (1996b). For selection of the C6VLCDR3 library, c-erbB-2 ECD concentrations of  $4.0 \times 10^{-8}$  M,  $1.0 \times 10^{-9}$  M,  $1.0 \times 10^{-10}$  M, and  $1.0 \times 10^{-11}$  M were used for selection rounds 1, 2, 3, and 4, respectively. The mixture of phage and antigen was gently rotated for one hour at room temperature and phage bound to biotinylated antigen captured using 100  $\mu$ l (round 1) or 50  $\mu$ l (rounds 2, 3, and 4) of streptavidin-coated M280 magnetic beads. After capture of phage, Dynabeads were washed a total of ten times (three times in PBS containing 0.05% Tween 20 (TPBS), twice in TPBS containing 2% skimmed milk powder (2% MTPBS), twice in PBS, once in 2% MPBS, and twice in PBS) using a Dynal magnetic particle concentrator. The Dynabeads were resuspended in 1 ml PBS, and 300  $\mu$ l were used to infect 10 ml log phase *E. coli* TG1 which were plated on TYE-AMP-Glu plates. For selection of the C6VHCDR3 libraries, c-erbB-2 ECD concentrations of  $5.0 \times 10^{-9}$  M,  $5.0 \times 10^{-11}$  M,  $5.0 \times 10^{-12}$  M, and  $5.0 \times 10^{-13}$  M were used for selection rounds 1, 2, 3, and 4, respectively and the phage captured by incubating with 50  $\mu$ l of Dynabeads for five minutes. The washing protocol was altered to select for scFv with the lowest  $k_{off}$  (Hawkins *et al.*, 1992). Dynabeads with bound phage were initially subjected to five rapid washes (4  $\times$  TPBS, 1  $\times$  MPBS) followed by six 30 minute incubations in one of three washing buffer (2  $\times$  TPBS, 2  $\times$  MPBS, 2  $\times$  PBS) containing  $1.0 \times 10^{-7}$  M c-erbB-2 ECD. Bound phage were eluted from the Dynabeads by sequential incubation with 100  $\mu$ l of 4 M  $MgCl_2$  for 15 minutes followed by 100  $\mu$ l of 100 mM HCl for five minutes. Eluates were combined and neutralized with 1.5 ml of 1 M Tris HCl (pH 7.5) and one third of the eluate used to infect log phase *E. coli* TG1.

### Initial scFv characterization

Initial analysis of selected scFv clones for binding to c-erbB-2 ECD was determined by phage ELISA. To prepare phage for ELISA, single ampicillin-resistant colonies were transferred into microtiter plate wells containing 100  $\mu$ l 2  $\times$  TY-AMP-0.1% glucose and grown for three hours at 37°C to an  $A_{600}$  of approximately 0.5. VCSML3 helper phage ( $2.5 \times 10^8$  phage) were added to each well, and the cells incubated for one hour at 37°C. Kanamycin was then added to each well to a final concentration of 25  $\mu$ g/ml and the bacteria grown overnight at 37°C. Supernatants containing phage were used for ELISA. For ELISA, Immulon 4 plates (Dynatech) were incubated overnight at 4°C with ImmunoPure avidin (10  $\mu$ g/ml in PBS; Pierce). After washing three times with PBS to remove unbound avidin, wells were incubated with biotinylated c-erbB-2 ECD as by Schier *et al.* (1995). Binding of scFv phage to c-erbB-2 ECD was detected with peroxidase-conjugated anti-ML3 antibody (Pharmacia) and ABTS (Sigma) as substrate. Selected binders were further characterized by DNA sequencing of the  $V_H$  and  $V_L$  genes (Sanger *et al.*, 1977).

Ranking of scFv by  $k_{off}$  was performed using SPR in a BIAcore as described by Schier *et al.* (1996b). Briefly, 10 ml cultures of 24 ELISA positive clones from the third and fourth round of selection were grown to an  $A_{600}$  of approximately 0.8, scFv expression induced (De Bellis & Schwartz, 1990) and the culture grown overnight at 25°C. scFv were harvested from the periplasm (Breitling *et al.*, 1991), and the periplasmic fraction dialyzed for 48 hours against Hepes buffered saline (10 mM Hepes, 150 mM NaCl, pH 7.4, HBS). In a BIAcore flow cell, approximately 1400 resonance units (RU) of c-erbB-2 ECD were coupled to a CM5 sensor chip (Schier *et al.*, 1996b) using NHS-EDC chemistry (Johnsson *et al.*, 1991). Association and dissociation of undiluted scFv in the periplasmic fraction were measured under a constant flow of 5  $\mu$ l/minute and HBS as running buffer. An apparent  $k_{off}$  was determined from the dissociation part of the sensorgram for each scFv analyzed (Karlsson *et al.*, 1991). The flow cell was regenerated between samples using sequential injections of 4 M  $MgCl_2$  and 100 mM triethylamine without significant change in the sensorgram baseline after analysis of more than 100 samples.

### Subcloning, expression and purification of scFv

To facilitate purification of scFv selected from the C6VLCDR3 library, the scFv genes were subcloned (Schier *et al.*, 1995) into the expression vector pUC 119 Sfi-NotmycHis, which results in the addition of a hexa-histidine tag at the C-terminal end of the scFv. The scFv selected from the C6VHCDR3 library already have a C-terminal hexa-histidine tag and therefore could be purified without subcloning. 500 ml cultures of *E. coli* TG1 harboring one of the C6.5 mutant phagemids were grown, expression of scFv induced (De Bellis & Schwartz, 1990), and the culture grown at 25°C overnight. scFv were harvested from the periplasm (Breitling *et al.*, 1991), dialyzed overnight at 4°C against eight liters of IMAC loading buffer (50 mM sodium phosphate (pH 7.5), 500 mM NaCl, 20 mM imidazole) and then filtered through a 0.2 micron filter.

scFv was purified by IMAC (Hochuli *et al.*, 1988) exactly as described by Schier *et al.* (1995). To separate monomeric, dimeric and aggregated scFv, samples were concentrated to a volume <1 ml in a Centricon 10

(Amicon) and fractionated on a Superdex 75 column using a running buffer of HBS. The purity of the final preparation was evaluated by assaying an aliquot by SDS-PAGE. Protein bands were detected by Coomassie staining. The concentration was determined spectrophotometrically, assuming an  $A_{280}$  nm of 1.0 corresponds to an scFv concentration of 0.7 mg/ml.

### Measurement of affinity and binding kinetics

The  $K_d$  of scFv were determined using SPR in a BIAcore (Schier *et al.*, 1996b). In a BIAcore flow cell, approximately 1400 RU of c-erbB-2 ECD (90 kDa, McCartney *et al.*, 1995) were coupled to a CM5 sensor chip (Johnsson *et al.*, 1991). Association rates were measured under continuous flow of 5  $\mu$ l/minute using concentrations ranging from  $5.0 \times 10^{-8}$  to  $8.0 \times 10^{-7}$  M.  $k_{on}$  was determined from a plot of  $(\ln(dR/dt))/t$  versus concentration (Karlsson *et al.*, 1991). To verify that differences in  $K_{on}$  were not due to differences in immunoreactivity, the relative concentrations of functional scFv were determined using SPR in a BIAcore (Karlsson *et al.*, 1993; Schier *et al.*, 1996b). Briefly, 4000 RU of c-erbB-2 ECD were coupled to a CM5 sensor chip and the rate of binding of C6.5 (RU/s) determined under a constant flow of 30  $\mu$ l/minute. Over the concentration range of  $1.0 \times 10^{-9}$  M to  $1.0 \times 10^{-7}$  M, the rate of binding was proportional to the log of the scFv concentration. Purified scFv were diluted to the same concentration ( $1.0 \times 10^{-8}$  M and  $2.0 \times 10^{-8}$  M) as determined by  $A_{280}$ . The rate of binding to c-erbB-2 ECD was measured and used to calculate the concentration based on the standard curve constructed from C6.5. Dissociation rates were measured using a constant flow of 25  $\mu$ l/minute and a scFv concentration of  $1.0 \times 10^{-6}$  M.  $k_{off}$  was determined during the first two minutes of dissociation for scFv mutated in  $V_L$  CDR3 (Karlsson *et al.*, 1991) and during the first 15 to 60 minutes for clones with  $k_{off}$  below  $5 \times 10^{-4}$  s $^{-1}$  (scFv mutated in  $V_H$  CDR3 and combined scFv). To exclude rebinding,  $k_{off}$  was determined in the presence and absence of  $5.0 \times 10^{-7}$  M c-erbB-2 ECD as described by Schier *et al.* (1996b).

### Cell surface retention assay

The cell surface retention of selected scFv was determined on live SK-OV-3 cells using a fluorescence activated cell sorter (FACS). Purified scFv were labeled with NHS-LC-Biotin (Pierce) using the manufacturers instructions. The concentration of immunoreactive biotinylated scFv was calculated using SPR as described above and by Schier *et al.* (1996b). The efficiency of biotinylation was also determined in a BIAcore using a flow cell to which 5000 RU of streptavidin was coupled. The total responses after association were compared between samples and concentrations of scFv were adjusted using the results obtained from the BIAcore. For the assay, aliquots of SK-OV-3 cells ( $1.2 \times 10^7$  c-erbB-2 positive cells) were incubated with 14  $\mu$ g biotinylated scFv in a total volume of 0.5 ml (1  $\mu$ M scFv) FACS buffer (PBS containing 1% BSA and 0.1%  $NaN_3$ ) for one hour at 37°C. Cells were washed twice with 10 ml FACS buffer (4°C) and resuspended in 12 ml FACS buffer and further incubated at 37°C. Aliquots of cells (0.5 ml from 12 ml containing  $5 \times 10^5$  cells) were taken after five minutes, every 15 minutes for the first hour and after two hours repeating the wash and resuspension cycle. Washed cell aliquots were fixed with 1% paraformaldehyde, washed

twice with FACS buffer, and incubated for 15 minutes at 4°C with a 1:800 dilution of phycoerythrin-labeled streptavidin (Pierce). Fluorescence was measured by FACS and the percent retained fluorescence on the cell surface plotted *versus* the time points. scFv used for the cell surface retention assay were C6.5 ( $K_d = 1.6 \times 10^{-8}$  M), C6ML3-9 ( $K_d = 1.0 \times 10^{-9}$  M), C6MH3-B1 ( $K_d = 1.2 \times 10^{-10}$  M), and the anti-digoxin scFv 26-10 (Huston *et al.*, 1988) as negative control.

### Construction of scFv combining higher affinity $V_H$ and $V_L$ genes

The  $V_L$  CDR3 gene sequences of the two highest affinity scFv isolated from the C6VLCDR3 library (C6ML3-9 or C6ML3-12) were combined with the highest affinity scFv previously obtained from light chain shuffling (C611,  $K_d = 2.5 \times 10^{-9}$  M; Schier *et al.*, 1996b). The C6L1 plasmid (10 ng/ $\mu$ l) was used as a template for PCR amplification using primers LMB3 and either PML3-9 or PML3-12 (Table 1). The gel purified PCR fragments were reamplified using primers LMB3 and H $\mu$  $\lambda$  2-3ForNot (Marks *et al.*, 1991) to introduce a *NotI* restriction site at the 3'-end of the scFv. The gel purified PCR fragments were digested with *NcoI* and *NotI* and ligated into pUC119 Sfi-NotmycHis digested with *NcoI* and *NotI*. The resulting scFv were designated C6-9L1 and C6-12L1. The  $V_L$  genes of C6-9L1 and C6-12L1 were combined with the  $V_H$  genes of the two highest affinity scFv from the C6VHCDR3 libraries (C6MH3-B1 and C6MH3-B47). The rearranged  $V_H$  genes of C6MH3-B1 and -B47 were amplified by PCR using the primer LMB3 and PC6VH1FOR (Schier *et al.*, 1996b), digested with *NcoI* and *XhoI* (located in FR4 of the heavy chain) and ligated into C6-9L1 or C6-12L1 digested with *NcoI* and *XhoI* to create C6-B1L1 and C6-B47L1. The heavy chain of C6MH3-B1 or C6MH3-B47 was amplified by PCR using LMB3 and one of the PCD primers (PCD1, PCD2, PCD3, PCD5, or PCD6; Table 1) to construct combinations of scFv from the C6VHCDR3B and D libraries. The purified PCR fragments were spliced with the  $V_L$  fragment of C6ML3-9 (VHD2) that was used to create the C6VHCDR3D library exactly as described above. The full length scFv gene was digested with *NcoI* and *NotI* and ligated into pUC119 Sfi-NotmycHis. Clones were termed C6-B1D1, -B1D2, -B1D3, -B1D5, -B1D6, -B47D1, -B47D2, -B47D3, -B47D5, and -B47D6. Colonies were screened for the presence of the correct insert by PCR fingerprinting and confirmed by DNA sequencing. scFv were expressed, purified, and affinities determined by SPR, as described above.

### High resolution functional scan of C6.5 $V_H$ CDR3

A high resolution functional scan of the C6.5  $V_H$  CDR3 was performed by individually mutating residues 95 to 99, 100a to 100d, and 100g to 102 to alanine. The pair of cysteine residues (100 and 100e) were simultaneously mutated to serine. Residue 100f (alanine) was not studied. Mutations were introduced by oligonucleotide directed mutagenesis using the method of Kunkel *et al.* (1987). Insertion of the correct mutation was verified by DNA sequencing, and scFv was expressed (De Bellis & Schwartz, 1990; Breitling *et al.*, 1991) and purified by IMAC (Hochuli *et al.*, 1988). Affinities were determined by SPR as described above (Schier *et al.*, 1996b) and compared to C6.5 scFv.

### Modeling of location of mutations

The location of mutations in mutated scFv was modeled on the structure of the Fab KOL (Marquart *et al.*, 1980) using the program MidasPlus (Ferrin *et al.*, 1988) on a Silicon Graphics workstation.

### Acknowledgements

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- [(sFv)<sub>2</sub>] with improved solution and targeting properties: anti-digoxin 26-10 (sFv)<sub>2</sub> and anti-c-erbB-2 741F8 (sFv)<sub>2</sub> made by protein folding and bonded through C-terminal cysteinyl peptides. *Protein Eng.* 8, 301-314.
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Appendix 6

Marks JD and Schier R. High affinity human antibodies to novel tumor antigens.  
Provisonal patent application.

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Atty. Docket No. 02307E-061410

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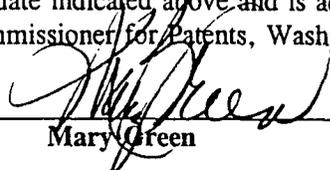
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**PATENT APPLICATION**  
**ASST. COMMISSIONER FOR PATENTS**  
**Washington, D. C. 20231**

Sir:

Transmitted herewith for filing is the  
 patent application of  
 design patent application of  
 continuation-in-part patent application of

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By   
Mary Green

Inventor(s): **James D. Marks and Robert Schier**

For: **NOVEL HIGH AFFINITY HUMAN ANTIBODIES TO TUMOR ANTIGENS**

This application claims priority from each of the following Application Nos./filing dates:  
60/000,238/filed June 14, 1995 ; 60/000,250 /June 15, 1995 ; \_\_\_\_\_ / \_\_\_\_\_

Please amend this application by adding the following before the first sentence: --This application claims the benefit of U.S. Provisional Application No. 60/\_\_\_\_\_, filed \_\_\_\_\_.--

Enclosed are:

- 5 sheet(s) of  formal  informal drawing(s).
- An assignment of the invention to \_\_\_\_\_
- A  signed  unsigned Declaration & Power of Attorney.
- A  signed  unsigned Declaration.
- A Power of Attorney by Assignee and Exclusion of Inventor(s) Under Rule 32/unsigned
- A verified statement to establish small entity status under 37 CFR 1.9 and 37 CFR 1.27  is enclosed  was filed in the earliest of the above-identified patent application(s).
- A certified copy of a \_\_\_\_\_ application.
- Information Disclosure Statement under 37 CFR 1.97.
- 

**In view of the Unsigned Declaration as filed with this application and pursuant to 37 CFR §1.53(d), Applicant requests deferral of the filing fee until submission of the Missing Parts of Application.**

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Page 2

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Dated: \_\_\_\_\_

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**DECLARATION**

As a below named inventor, I declare that:

My residence, post office address and citizenship are as stated below next to my name; I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural inventors are named below) of the subject matter which is claimed and for which a patent is sought on the invention entitled: **NOVEL HIGH AFFINITY HUMAN ANTIBODIES TO TUMOR ANTIGENS** the specification of which  X  is attached hereto or \_\_\_ was filed on \_\_\_\_\_ as Application No. \_\_\_\_\_ and was amended on \_\_\_ (if applicable).

I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, Section 1.56. I claim foreign priority benefits under Title 35, United States Code, Section 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed.

**Prior Foreign Application(s)**

Country	Application No.	Date of Filing	Priority Claimed Under 35 USC 119
			Yes ___ No ___
			Yes ___ No ___

I hereby claim the benefit under Title 35, United States Code § 119(e) of any United States provisional application(s) listed below:

Application No.	Filing Date
60/000,238	June 14, 1995
60/000,250	June 15, 1995

I claim the benefit under Title 35, United States Code, Section 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, section 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, section 1.56 which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

Application No.	Date of Filing	Status
		___ Patented ___ Pending ___ Abandoned
		___ Patented ___ Pending ___ Abandoned

Full Name of Inventor 1	Last Name Marks	First Name James	Middle Name or Initial D.	
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Post Office Address	Post Office Address	City	State/Country	Zip Code

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Signature of Inventor 1	Signature of Inventor 2	Signature of Inventor 3
James D. Marks	Robert Schier	
Date	Date	Date

**PATENT APPLICATION**

**NOVEL HIGH AFFINITY HUMAN ANTIBODIES TO TUMOR  
ANTIGENS**

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~~CONFIDENTIAL~~

**NOVEL HIGH AFFINITY HUMAN ANTIBODIES TO TUMOR  
ANTIGENS**

5

**BACKGROUND OF THE INVENTION**

This application is a continuation-in-part of United States Provisional Applications U.S.S.N. 60/000,238 and U.S.S.N. 60/000,250, filed on June 14, 1995 and June 15, 1995 respectively. These applications are incorporated by reference for all purposes.

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The invention was made by or under a contract with the following agencies of the United States Government: Army Grant No. DAMD17-94-J-4433 and the Department of Health and Human Services, National Institutes of Health, Grant No. U01 CA51880.

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This invention pertains to the fields of immunodiagnostics and immunotherapeutics. In particular, this invention pertains to the discovery of novel human antibodies that specifically bind to c-erbB-2, and to chimeric molecules containing these antibodies.

20

Conventional cancer chemotherapeutic agents cannot distinguish between normal cells and tumor cells and hence damage and kill normal proliferating tissues. One approach to reduce this toxic side effect is to specifically target the chemotherapeutic agent to the tumor. This is the rationale behind the development of immunotoxins, chimeric molecules composed of an antibody either chemically conjugated or fused to a toxin that binds specifically to antigens on the surface of a tumor cell thereby killing or inhibiting the growth of the cell (Frankel *et al. Ann. Rev. Med.*, 37: 127 (1986)). The majority of immunotoxins prepared to date, have been made using murine monoclonal antibodies (Mabs) that exhibit specificity for tumor cells. Immunotoxins made from Mabs demonstrate relatively selective killing of tumor cells *in vitro* and tumor regression in animal models (*id.*).

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Despite these promising results, the use of immunotoxins in humans has been limited by toxicity, immunogenicity and a failure to identify highly specific tumor antigens (Byers *et al. Cancer Res.*, 49: 6153). Nonspecific toxicity results from the

failure of the monoclonal antibody to bind specifically and with high affinity to tumor cells. As a result, nonspecific cell killing occurs. In addition, the foreign immunotoxin molecule elicits a strong immune response in humans. The immunogenicity of the toxin portion of the immunotoxin has recently been overcome by using the human analog of RNase (Rybak *et al. Proc. Nat. Acad. Sci., USA*, 89: 3165 (1992)). The murine antibody portion, however, is still significantly immunogenic (Sawler *et al., J. Immunol.*, 135: 1530 (1985)).

Immunogenicity could be avoided and toxicity reduced if high affinity tumor specific human antibodies were available. However, the production of human monoclonal antibodies using conventional hybridoma technology has proven extremely difficult (James *et al., J. Immunol. Meth.*, 100: 5 (1987)). Furthermore, the paucity of purified tumor-specific antigens makes it necessary to immunize with intact tumor cells or partially purified antigen. Most of the antibodies produced react with antigens which are also common to normal cells and are therefore unsuitable for use as tumor-specific targeting molecules.

#### SUMMARY OF THE INVENTION

This invention provides novel human antibodies that specifically bind to the extracellular domain of the c-erbB-2 protein product of the HER2/neu oncogene. This antigen (marker) is overexpressed on many cancers (*e.g.* carcinomas) and thus the antibodies of the present invention specifically bind to tumor cells that express c-erbB-2.

In a preferred embodiment, the antibody is a C6 antibody derived from the sFv antibody C6.5. The antibody may contain a variable heavy chain, a variable light chain, or both a variable heavy and variable light chain of C6.5 or its derivatives. In addition the antibody may contain a variable heavy chain, a variable light chain or both a variable heavy and variable light chain of C6.5 in which one or more of the variable heavy or variable light complementarity determining regions (CDR1, CDR2 or CDR3) has been altered (*e.g.*, mutated). Particularly preferred CDR variants are listed in the specification and in Examples 1, 2 and 3. Particularly preferred C6 antibodies include C6.5, C6ML3-14, C6L-1 and C6MH3-B1. In various preferred embodiments, these antibodies are single chain antibodies (sFv also known as scFv) comprising a variable heavy chain joined to a variable light chain either directly or through a peptide linker. Other preferred embodiments of the C6 antibodies and C6.5, C6ML3-14, C6L1, and C6MH3-B1, in particular, include Fab, the dimer (Fab')<sub>2</sub>, and the dimer (sFv')<sub>2</sub>.

Particularly preferred (sFv')<sub>2</sub> dimers are fusion proteins where the Sfv' components are joined through a peptide linkage or through a peptide (G<sub>4</sub>S). Still other preferred C6 antibodies include an antibody selected from the group consisting of an antibody having a V<sub>L</sub> domain with one of the amino acid sequences shown in Table 10, an antibody having a V<sub>H</sub> domain with one of the amino acid sequences shown in Table 12, an antibody having a V<sub>L</sub> CDR3 domain having one of the amino acid sequences shown in Tables 4, 15, and 16, and an antibody having a V<sub>H</sub> CDR3 domain having one of the amino acid sequences shown in Tables 13 and 14. Other preferred embodiments are to be found replete throughout the specification.

10 In a particularly preferred embodiment, the C6 antibody has a K<sub>d</sub> ranging from about 1.6 x 10<sup>-8</sup> to about 1 x 10<sup>-12</sup> M in SK-BR-3 cells using Scatchard analysis or as measured against purified c-erbB-2 by surface plasmon resonance in a BIAcore.

In another embodiment the present invention provides for nucleic acids that encode any of the above-described C6 antibodies. The invention also provides for nucleic acids that encode the amino acid sequences of C6.5, C6ML3-14, C6L1, C6MH3-B1, or any of the other amino acid sequences encoding C6 antibodies and described in Example 1, 2 or 3. In addition this invention provides for nucleic acid sequences encoding any of these amino acid sequences having conservative amino acid substitutions.

20 In still another embodiment, this invention provides for proteins comprising one or more complementarity determining regions selected from the group consisting of the complementarity determining regions of Tables 10, 12, 13, 14, 15, and 16 and of any of the examples, in particular of Examples 1, 2 or 3. Other particularly preferred antibodies include any of the antibodies expressed by the clones described herein.

In still yet another embodiment, this invention provides for cells comprising a recombinant nucleic acid which is any of the above described nucleic acids.

This invention also provides for chimeric molecules that specifically bind a tumor cell bearing c-erbB-2. The chimeric molecule comprises an effector molecule joined to any of the above-described C6 antibodies. In a preferred embodiment, the effector molecule is selected from the group consisting of a cytotoxin (e.g. PE, DT, Ricin A, *etc.*), a label, a radionuclide, a drug, a liposome, a ligand, an antibody, and an

antigen binding domaine). The C6 antibody may be chemically conjugated to the effector molecule or the chimeric molecule may be expressed as a fusion protein.

This invention provides for methods of making C6 antibodies. One method proceeds by i) providing a phage library presenting a C6.5 variable heavy chain and a multiplicity of human variable light chains; ii) panning the phage library on c-erbB-2; and iii) isolating phage that specifically bind c-erbB-2. This method optionally further includes iv) providing a phage library presenting the variable light chain of the phage isolated in step iii and a multiplicity of human variable heavy chains; v) panning the phage library on c-erbB-2; and vi) isolating phage that specifically bind c-erbB-2.

Another method for making a C6 antibody proceeds by i) providing a phage library presenting a C6.5 variable light chain and a multiplicity of human variable heavy chains; ii) panning the phage library on c-erbB-2; and iii) isolating phage that specifically bind c-erbB-2.

Yet another method for making a C6 antibody involves i) providing a phage library presenting a C6.5 variable light and a C6.5 variable heavy chain encoded by a nucleic acid variable in the sequence encoding CDR1, CDR2 or CDR3 such that each phage displays a different CDR; ii) panning the phage library on c-erbB-2; and isolating the phage that specifically bind c-erbB-2.

This invention also provides a method for impairing growth of tumor cells bearing c-erbB-2. This method involves contacting the tumor with a chimeric molecule comprising a cytotoxin attached to a human C6 antibody that specifically binds c-erbB-2.

Finally, this invention provides a method for detecting tumor cells bearing c-erbB-2. This method involves contacting the biological samples derived from a tumor with a chimeric molecule comprising a label attached to a human C6 antibody that specifically binds c-erbB-2.

### Definitions

The following abbreviations are used herein: AMP, ampicillin; c-erbB-2 ECD, extracellular domain of c-erbB-2; CDR, complementarity determining region; ELISA, enzyme linked immunosorbent assay; FACS, fluorescence activated cell sorter; FR, framework region; Glu, glucose; HBS, hepes buffered saline, 10 mM hepes, 150 mM NaCl, pH 7.4; IMAC, immobilized metal affinity chromatography;  $k_{on}$ , association rate constant;  $k_{off}$ , dissociation rate constant; MPBS, skimmed milk powder in PBS;

MTPBS, skimmed milk powder in TPBS; PBS, phosphate buffered saline, 25 mM  $\text{NaH}_2\text{PO}_4$ , 125 mM NaCl, pH 7.0; PCR, polymerase chain reaction; RU, resonance units; scFv or sFv, single-chain Fv fragment; sFv': Fv containing cysteine; TPBS, 0.05% v/v Tween 20 in PBS; SPR, surface plasmon resonance;  $V_{\kappa}$ , immunoglobulin kappa light chain variable region;  $V_{\lambda}$ , immunoglobulin lambda light chain variable region;  $V_L$ , immunoglobulin light chain variable region;  $V_H$ , immunoglobulin heavy chain variable region; wt, wild type.

As used herein, an "antibody" refers to a protein consisting of one or more polypeptides substantially encoded by immunoglobulin genes or fragments of immunoglobulin genes. The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon and mu constant region genes, as well as myriad immunoglobulin variable region genes. Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD and IgE, respectively.

A typical immunoglobulin (antibody) structural unit is known to comprise a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25 kD) and one "heavy" chain (about 50-70 kD). The N-terminus of each chain defines a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The terms variable light chain ( $V_L$ ) and variable heavy chain ( $V_H$ ) refer to these light and heavy chains respectively.

Antibodies exist as intact immunoglobulins or as a number of well characterized fragments produced by digestion with various peptidases. Thus, for example, pepsin digests an antibody below the disulfide linkages in the hinge region to produce  $\text{F(ab)'}_2$ , a dimer of Fab which itself is a light chain joined to  $V_H\text{-C}_H1$  by a disulfide bond. The  $\text{F(ab)'}_2$  may be reduced under mild conditions to break the disulfide linkage in the hinge region thereby converting the  $(\text{Fab}')_2$  dimer into an Fab' monomer. The Fab' monomer is essentially an Fab with part of the hinge region (*see, Fundamental Immunology*, W.E. Paul, ed., Raven Press, N.Y. (1993), for a more detailed description of other antibody fragments). While various antibody fragments are defined in terms of the digestion of an intact antibody, one of skill will appreciate that such Fab' fragments may be synthesized *de novo* either chemically or by utilizing recombinant DNA methodology. Thus, the term antibody, as used herein also includes antibody fragments either produced by the modification of whole antibodies or synthesized *de novo* using

recombinant DNA methodologies. Preferred antibodies include single chain antibodies, more preferably single chain Fv (sFv) antibodies in which a variable heavy and a variable light chain are joined together (directly or through a peptide linker) to form a continuous polypeptide.

5                   An "antigen-binding site" or "binding portion" refers to the part of an immunoglobulin molecule that participates in antigen binding. The antigen binding site is formed by amino acid residues of the N-terminal variable ("V") regions of the heavy ("H") and light ("L") chains. Three highly divergent stretches within the V regions of the heavy and light chains are referred to as "hypervariable regions" which are  
10                   interposed between more conserved flanking stretches known as "framework regions" or "FRs". Thus, the term "FR" refers to amino acid sequences which are naturally found between and adjacent to hypervariable regions in immunoglobulins. In an antibody molecule, the three hypervariable regions of a light chain and the three hypervariable regions of a heavy chain are disposed relative to each other in three dimensional space to  
15                   form an antigen binding "surface". This surface mediates recognition and binding of the target antigen. The three hypervariable regions of each of the heavy and light chains are referred to as "complementarity determining regions" or "CDRs" and are characterized, for example by Kabat *et al. Sequences of proteins of immunological interest*, 4th ed. U.S. Dept. Health and Human Services, Public Health Services, Bethesda, MD (1987).

20                   As used herein, the terms "immunological binding" and "immunological binding properties" refer to the non-covalent interactions of the type which occur between an immunoglobulin molecule and an antigen for which the immunoglobulin is specific. The strength or affinity of immunological binding interactions can be expressed in terms of the dissociation constant ( $K_d$ ) of the interaction, wherein a smaller  $K_d$   
25                   represents a greater affinity. Immunological binding properties of selected polypeptides can be quantified using methods well known in the art. One such method entails measuring the rates of antigen-binding site/antigen complex formation and dissociation, wherein those rates depend on the concentrations of the complex partners, the affinity of the interaction, and on geometric parameters that equally influence the rate in both  
30                   directions. Thus, both the "on rate constant" ( $k_{on}$ ) and the "off rate constant" ( $k_{off}$ ) can be determined by calculation of the concentrations and the actual rates of association and dissociation. The ratio of  $k_{off}/k_{on}$  enables cancellation of all parameters not related to

affinity and is thus equal to the dissociation constant  $K_d$ . *See, generally, Davies et al. Ann. Rev. Biochem., 59: 439-473 (1990).*

The term "C6 antibody", as used herein refers to antibodies derived from C6.5 whose sequence is expressly provided herein. C6 antibodies preferably have a binding affinity of about  $1.6 \times 10^8$  or better and are preferably derived by screening (for affinity to c-erbB-2) a phage display library in which a known C6 variable heavy ( $V_H$ ) chain is expressed in combination with a multiplicity of variable light ( $V_L$ ) chains or conversely a known C6 variable light chain is expressed in combination with a multiplicity of variable heavy ( $V_H$ ) chains. C6 antibodies also include those antibodies produced by the introduction of mutations into the variable heavy or variable light complementarity determining regions (CDR1, CDR2 or CDR3) as described herein. Finally C6 antibodies include those antibodies produced by any combination of these modification methods as applied to C6.5 and its derivatives.

A single chain Fv ("sFv" or "scFv") polypeptide is a covalently linked  $V_H::V_L$  heterodimer which may be expressed from a nucleic acid including  $V_H$ - and  $V_L$ -encoding sequences either joined directly or joined by a peptide-encoding linker. Huston, *et al. Proc. Nat. Acad. Sci. USA*, 85: 5879-5883 (1988). A number of structures for converting the naturally aggregated-- but chemically separated light and heavy polypeptide chains from an antibody V region into an sFv molecule which will fold into a three dimensional structure substantially similar to the structure of an antigen-binding site. *See, e.g. U.S. Patent Nos. 5, 091,513 and 5,132,405 and 4,956,778.*

In one class of embodiments, recombinant design methods can be used to develop suitable chemical structures (linkers) for converting two naturally associated--but chemically separate--heavy and light polypeptide chains from an antibody variable region into a sFv molecule which will fold into a three-dimensional structure that is substantially similar to native antibody structure.

Design criteria include determination of the appropriate length to span the distance between the C-terminal of one chain and the N-terminal of the other, wherein the linker is generally formed from small hydrophilic amino acid residues that do not tend to coil or form secondary structures. Such methods have been described in the art. *See, e.g., U.S. Patent Nos. 5,091,513 and 5,132,405 to Huston et al.; and U.S. Patent No. 4,946,778 to Ladner et al.*

In this regard, the first general step of linker design involves identification of plausible sites to be linked. Appropriate linkage sites on each of the  $V_H$  and  $V_L$  polypeptide domains include those which will result in the minimum loss of residues from the polypeptide domains, and which will necessitate a linker comprising a minimum number of residues consistent with the need for molecule stability. A pair of sites defines a "gap" to be linked. Linkers connecting the C-terminus of one domain to the N-terminus of the next generally comprise hydrophilic amino acids which assume an unstructured configuration in physiological solutions and preferably are free of residues having large side groups which might interfere with proper folding of the  $V_H$  and  $V_L$  chains. Thus, suitable linkers under the invention generally comprise polypeptide chains of alternating sets of glycine and serine residues, and may include glutamic acid and lysine residues inserted to enhance solubility. One particular linker under the invention has the amino acid sequence  $[(Gly)_4Ser]_3$ . Another particularly preferred linker has the amino acid sequence comprising 2 or 3 repeats of  $[(Ser)_4Gly]$  such as  $[(Ser)_4Gly]_3$ . Nucleotide sequences encoding such linker moieties can be readily provided using various oligonucleotide synthesis techniques known in the art. See, e.g., Sambrook, *supra*.

The phrase "specifically binds to a protein" or "specifically immunoreactive with", when referring to an antibody refers to a binding reaction which is determinative of the presence of the protein in the presence of a heterogeneous population of proteins and other biologics. Thus, under designated immunoassay conditions, the specified antibodies bind to a particular protein and do not bind in a significant amount to other proteins present in the sample. Specific binding to a protein under such conditions may require an antibody that is selected for its specificity for a particular protein. For example, C6 antibodies can be raised to the c-erbB-2 protein that bind c-erbB-2 and not to other proteins present in a tissue sample. A variety of immunoassay formats may be used to select antibodies specifically immunoreactive with a particular protein. For example, solid-phase ELISA immunoassays are routinely used to select monoclonal antibodies specifically immunoreactive with a protein. See Harlow and Lane (1988) *Antibodies, A Laboratory Manual*, Cold Spring Harbor Publications, New York, for a description of immunoassay formats and conditions that can be used to determine specific immunoreactivity.

A chimeric molecule is a molecule in which two or more molecules that exist separately in their native state are joined together to form a single molecule having the desired functionality of all of its constituent molecules. While the chimeric molecule may be prepared by covalently linking two molecules each synthesized separately, one of skill in the art will appreciate that where the chimeric molecule is a fusion protein, the chimera may be prepared de novo as a single "joined" molecule.

The term "conservative substitution" is used in reference to proteins or peptides to reflect amino acid substitutions that do not substantially alter the activity (specificity or binding affinity) of the molecule. Typically conservative amino acid substitutions involve substitution one amino acid for another amino acid with similar chemical properties (*e.g.* charge or hydrophobicity). The following six groups each contain amino acids that are typical conservative substitutions for one another:

- 1) Alanine (A), Serine (S), Threonine (T);
- 2) Aspartic acid (D), Glutamic acid (E);
- 3) Asparagine (N), Glutamine (Q);
- 4) Arginine (R), Lysine (K);
- 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and
- 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

## BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates the nucleic and amino acid sequence of the C6 sFv antibody C6.5.

Figure 2 shows the location of mutations in a light chain shuffled C6L1 and heavy chain shuffled C6H2 sFv. Mutations are indicated as shaded spheres on the  $\alpha$ -carbon tracing of the Fv fragment of the immunoglobulin KOL (Marquart *et al.* (1990). H1, H2, H3, L1, L2 and L3 refer to the  $V_H$  and  $V_L$  antigen binding loops respectively. Mutations in C6L1 are all located in the  $V_L$  domain with parental  $V_H$  sequence, mutations in C6H2 are all located in the  $V_H$  domain with parental  $V_L$  sequence. C6L1 has no mutations located in a  $\beta$ -strand which forms part of the  $V_H$ - $V_L$  interface. C6H2 has 2 conservative mutations located in the  $\beta$ -strand formed by framework 3 residues.

Figure 3 shows the locations of mutations in light chain shuffled sFv which spontaneously form dimers. Mutations are indicated as shaded spheres on the  $\alpha$ -

carbon tracing of the Fv fragment of the immunoglobulin KOL (Marquart et al., 1980) with the V<sub>L</sub> domain located on the left side of each panel. A=C6VLD; B=C6VLE; C=CdVLB; D=C6VLF. Each shuffled sFv has at least 1 mutation located in a β-strand which forms part of the V<sub>H</sub>-V<sub>L</sub> interface.

5                    Figure 4 illustrates the 72 hour biodistribution of a C6.5 diabody in SK-OV-3 tumor-bearing scid mice.

#### DETAILED DESCRIPTION

10                    This invention provides for novel human antibodies that specifically bind to the extracellular domain of the c-erbB-2 protein product of the HER2/neu oncogene. The c-erbB-2 marker is overexpressed by 30-50% of breast carcinomas and other adenocarcinomas and thus provides a suitable cell surface marker for specifically targeting tumor cells such as carcinomas. In contrast to previous known anti-cerbB-2 antibodies, the antibodies of the present invention (designated herein as C6 antibodies) are fully human antibodies. Thus, administration of these antibodies to a human host  
15                    elicits a little or no immunogenic response.

                         This invention additionally provides for chimeric molecules comprising the C6 antibodies of the present invention joined to an effector molecule. The C6 antibodies act as a "targeting molecule" that serves to specifically bind the chimeric molecule to  
20                    cells bearing the c-erbB-2 marker thereby delivering the effector molecule to the target cell.

                         An effector molecule typically has a characteristic activity that is desired to be delivered to the target cell (*e.g.* a tumor overexpressing c-erbB-2). Effector molecules include cytotoxins, labels, radionuclides, ligands, antibodies, drugs, liposomes,  
25                    and viral coat proteins that render the virus capable of infecting a c-erbB-2 expressing cell. Once delivered to the target, the effector molecule exerts its characteristic activity.

                         For example, in one embodiment, where the effector molecule is a cytotoxin, the chimeric molecule acts as a potent cell-killing agent specifically targeting the cytotoxin to tumor cells bearing the c-erbB-2 marker. Chimeric cytotoxins that  
30                    specifically target tumor cells are well known to those of skill in the art (*see, for example, Pastan et al., Ann. Rev. Biochem., 61: 331-354 (1992)*).

                         In another embodiment, the chimeric molecule may be used for detecting the presence or absence of tumor cells *in vivo* or *in vitro* or for localizing tumor cells *in*

*vivo*. These methods involve providing a chimeric molecule comprising an effector molecule, that is a detectable label attached to the C6 antibody. The C6 antibody specifically binds the chimeric molecule to tumor cells expressing the c-erbB-2 marker which are then marked by their association with the detectable label. Subsequent  
5 detection of the cell-associated label indicates the presence and/or location of a tumor cell.

In yet another embodiment, the effector molecule may be another specific binding moiety including, but not limited to an antibody, an antigen binding domain, a growth factor, or a ligand. The chimeric molecule will then act as a highly specific  
10 bifunctional linker. This linker may act to bind and enhance the interaction between cells or cellular components to which the chimeric protein binds. Thus, for example, where the "effector" component is an anti-receptor antibody or antibody fragment, the C6 antibody component specifically binds c-erbB-2 bearing cancer cells, while the effector component binds receptors (*e.g.*, IL-2, IL-4, Fc $\gamma$ I, Fc $\gamma$ II and Fc $\gamma$ III receptors) on the  
15 surface of immune cells. The chimeric molecule may thus act to enhance and direct an immune response toward target cancer cells.

In still yet another embodiment the effector molecule may be a pharmacological agent (*e.g.* a drug) or a vehicle containing a pharmacological agent. This is particularly suitable where it is merely desired to invoke a non-lethal biological  
20 response. Thus the C6 antibody receptor may be conjugated to a drug such as vinblastine, vindesine, melphalan, N-Acetylmelphalan, methotrexate, aminopterin, doxorubicin, daunorubicin, genistein (a tyrosine kinase inhibitor), an antisense molecule, and other pharmacological agents known to those of skill in the art, thereby specifically targeting the pharmacological agent to tumor cells expressing c-erbB-2.

25 Alternatively, the C6 antibody may be bound to a vehicle containing the therapeutic composition. Such vehicles include, but are not limited to liposomes, micelles, various synthetic beads, and the like.

One of skill in the art will appreciate that the chimeric molecules of the present invention optionally includes multiple targeting moieties bound to a single  
30 effector or conversely, multiple effector molecules bound to a single targeting moiety. In still other embodiment, the chimeric molecules includes both multiple targeting moieties and multiple effector molecules. Thus, for example, this invention provides for "dual targeted" cytotoxic chimeric molecules in which the C6 antibody is attached to a

cytotoxic molecule while another molecule (*e.g.* an antibody, or another ligand) is attached to the other terminus of the toxin. Such a dual-targeted cytotoxin might comprise, *e.g.* a C6 antibody substituted for domain Ia at the amino terminus of a PE and anti-TAC(Fv) inserted in domain III. Other antibodies may also be suitable effector molecules.

#### I. Preparation of C6 Antibodies.

The C6 antibodies of this invention are prepared using standard techniques well known to those of skill in the art in combination with the polypeptide and nucleic acid sequences provided herein. The polypeptide sequences may be used to determine appropriate nucleic acid sequences encoding the particular C6 antibody disclosed thereby. The nucleic acid sequence may be optimized to reflect particular codon "preferences" for various expression systems according to standard methods well known to those of skill in the art. Alternatively, the nucleic acid sequences provided herein may also be used to express C6 antibodies.

Using the sequence information provided, the nucleic acids may be synthesized according to a number of standard methods known to those of skill in the art. Oligonucleotide synthesis, is preferably carried out on commercially available solid phase oligonucleotide synthesis machines (Needham-VanDevanter *et al.* (1984) *Nucleic Acids Res.* 12:6159-6168) or manually synthesized using the solid phase phosphoramidite triester method described by Beaucage *et al.* (Beaucage *et al.* (1981) *Tetrahedron Letts.* 22(20): 1859-1862).

Once a nucleic acid encoding a C6 antibody is synthesized it may be amplified and/or cloned according to standard methods. Molecular cloning techniques to achieve these ends are known in the art. A wide variety of cloning and *in vitro* amplification methods suitable for the construction of recombinant nucleic acids, *e.g.*, encoding C6 antibody genes, are known to persons of skill. Examples of these techniques and instructions sufficient to direct persons of skill through many cloning exercises are found in Berger and Kimmel, *Guide to Molecular Cloning Techniques, Methods in Enzymology* volume 152 Academic Press, Inc., San Diego, CA (Berger); Sambrook *et al.* (1989) *Molecular Cloning - A Laboratory Manual* (2nd ed.) Vol. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor Press, NY, (Sambrook); and *Current Protocols in Molecular Biology*, F.M. Ausubel *et al.*, eds., Current Protocols, a

joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., (1994 Supplement) (Ausubel). Methods of producing recombinant immunoglobulins are also known in the art. See, Cabilly, U.S. Patent No. 4,816,567; and Queen *et al.* (1989) *Proc. Nat'l Acad. Sci. USA* 86: 10029-10033.

5                   Examples of techniques sufficient to direct persons of skill through *in vitro* amplification methods, including the polymerase chain reaction (PCR) the ligase chain reaction (LCR), Q $\beta$ -replicase amplification and other RNA polymerase mediated techniques are found in Berger, Sambrook, and Ausubel, as well as Mullis *et al.*, (1987) U.S. Patent No. 4,683,202; *PCR Protocols A Guide to Methods and Applications* (Innis *et al.* eds) Academic Press Inc. San Diego, CA (1990) (Innis); Arnheim & Levinson (October 1, 1990) *C&EN* 36-47; *The Journal Of NIH Research* (1991) 3, 81-94; (Kwoh *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86, 1173; Guatelli *et al.* (1990) *Proc. Natl. Acad. Sci. USA* 87, 1874; Lomell *et al.* (1989) *J. Clin. Chem* 35, 1826; Landegren *et al.*, (1988) *Science* 241, 1077-1080; Van Brunt (1990) *Biotechnology* 8, 291-294; Wu and Wallace, (1989) *Gene* 4, 560; and Barringer *et al.* (1990) *Gene* 89, 117. Improved methods of cloning *in vitro* amplified nucleic acids are described in Wallace *et al.*, U.S. Pat. No. 5,426,039.

                  Once the nucleic acid for a C6 antibody is isolated and cloned, one may express the gene in a variety of recombinantly engineered cells known to those of skill in the art. Examples of such cells include bacteria, yeast, filamentous fungi, insect (especially employing baculoviral vectors), and mammalian cells. It is expected that those of skill in the art are knowledgeable in the numerous expression systems available for expression of C6 antibodies.

                  In brief summary, the expression of natural or synthetic nucleic acids encoding C6 antibodies will typically be achieved by operably linking a nucleic acid encoding the antibody to a promoter (which is either constitutive or inducible), and incorporating the construct into an expression vector. The vectors can be suitable for replication and integration in prokaryotes, eukaryotes, or both. Typical cloning vectors contain transcription and translation terminators, initiation sequences, and promoters useful for regulation of the expression of the nucleic acid encoding the C6 antibody. The vectors optionally comprise generic expression cassettes containing at least one independent terminator sequence, sequences permitting replication of the cassette in both

eukaryotes and prokaryotes, *i.e.*, shuttle vectors, and selection markers for both prokaryotic and eukaryotic systems. *See* Sambrook.

To obtain high levels of expression of a cloned nucleic acid it is common to construct expression plasmids which typically contain a strong promoter to direct transcription, a ribosome binding site for translational initiation, and a transcription/translation terminator. Examples of regulatory regions suitable for this purpose in *E. coli* are the promoter and operator region of the *E. coli* tryptophan biosynthetic pathway as described by Yanofsky, C., 1984, *J. Bacteriol.*, 158:1018-1024 and the leftward promoter of phage lambda ( $P_L$ ) as described by Herskowitz and Hagen, 1980, *Ann. Rev. Genet.*, 14:399-445. The inclusion of selection markers in DNA vectors transformed in *E. coli* is also useful. Examples of such markers include genes specifying resistance to ampicillin, tetracycline, or chloramphenicol. *See* Sambrook for details concerning selection markers, *e.g.*, for use in *E. coli*.

Expression systems for expressing C6 antibodies are available using *E. coli*, *Bacillus sp.* (Palva *et al.* (1983) *Gene* 22:229-235; Mosbach *et al.*, *Nature*, 302:543-545 and *Salmonella. E. coli* systems are preferred.

The C6 antibodies produced by prokaryotic cells may require exposure to chaotropic agents for proper folding. During purification from, *e.g.*, *E. coli*, the expressed protein is optionally denatured and then renatured. This is accomplished, *e.g.*, by solubilizing the bacterially produced antibodies in a chaotropic agent such as guanidine HCl. The antibody is then renatured, either by slow dialysis or by gel filtration. *See*, U.S. Patent No. 4,511,503.

Methods of transfecting and expressing genes in mammalian cells are known in the art. Transducing cells with nucleic acids can involve, for example, incubating viral vectors containing C6 nucleic acids with cells within the host range of the vector. *See, e.g.*, *Methods in Enzymology*, vol. 185, Academic Press, Inc., San Diego, CA (D.V. Goeddel, ed.) (1990) or M. Krieger, *Gene Transfer and Expression -- A Laboratory Manual*, Stockton Press, New York, NY, (1990) and the references cited therein.

The culture of cells used in the present invention, including cell lines and cultured cells from tissue or blood samples is well known in the art. Freshney (*Culture of Animal Cells, a Manual of Basic Technique, third edition* Wiley-Liss, New York (1994)) and the references cited therein provides a general guide to the culture of cells.

Techniques for using and manipulating antibodies are found in Coligan (1991) *Current Protocols in Immunology* Wiley/Greene, NY; Harlow and Lane (1989) *Antibodies: A Laboratory Manual* Cold Spring Harbor Press, NY; Stites *et al.* (eds.) *Basic and Clinical Immunology* (4th ed.) Lange Medical Publications, Los Altos, CA, and references cited therein; Goding (1986) *Monoclonal Antibodies: Principles and Practice* (2d ed.) Academic Press, New York, NY; and Kohler and Milstein (1975) *Nature* 256: 495-497. C6 antibodies which are specific for *c-erbB-2* bind *c-erbB-2* and have a  $K_D$  of  $1\mu\text{M}$  or better, with preferred embodiments having a  $K_D$  of 1 nM or better and most preferred embodiments having a  $K_D$  of 0.1 nM or better.

In a preferred embodiment the C6 antibody gene (*e.g.* C6.5 sFv gene) is subcloned into the expression vector pUC119Sfi/NotHismyc, which is identical to the vector described by Griffiths *et al.*, *EMBO J.*, 13: 3245-3260 (1994), (except for the elimination of an XbaI restriction site). This results in the addition of a hexa-histidine tag at the C-terminal end of the sFv. A pHEN-1 vector DNA containing the C6.5 sFv DNA is prepared by alkaline lysis miniprep, digested with NcoI and NotI, and the sFv DNA purified on a 1.5% agarose gel. The C6 sFv DNA is ligated into pUC119Sfi1/Not1Hismyc digested with NcoI and NotI and the ligation mixture used to transform electrocompetent *E.coli* HB2151. For expression, 200 ml of 2 x TY media containing 100 mg/ml ampicillin and 0.1% glucose is inoculated with *E.coli* HB2151 harboring the C6 gene in pUC119Sfi1/Not1Hismyc. The culture is grown at 37°C to an A600 nm of 0.8. Soluble sFv is expression induced by the addition of IPTG to a final concentration of 1 mM, and the culture is grown at 30° C in a shaker flask overnight.

The C6 sFv may then be harvested from the periplasm using the following protocol: Cells are harvested by centrifugation at 4000 g for 15 min, resuspended in 10 ml of ice cold 30 mM Tris-HCl pH 8.0, 1 mM EDTA, 20% sucrose, and incubated on ice for 20 minutes. The bacteria are then pelleted by centrifugation at 6000 g for 15 min. and the "periplasmic fraction" cleared by centrifugation at 30,000g for 20 min. The supernatant is then dialyzed overnight at 4°C against 8 L of IMAC loading buffer (50 mM sodium phosphate pH 7.5, 500 mM NaCl, 20 mM imidazole) and then filtered through a 0.2 micron filter.

In a preferred embodiment, the C6 sFv is purified by IMAC. All steps are performed at 4°C. A column containing 2 ml of Ni-NTA resin (Qiagen) is washed with 20 ml IMAC column wash buffer (50 mM sodium phosphate pH 7.5, 500 mM

NaCl, 250 mM imidazole) and 20 ml of IMAC loading buffer. The periplasmic preparation is then loaded onto the column and the column washed sequentially with 50 ml IMAC loading buffer and 50 ml IMAC washing buffer (50 mM sodium phosphate pH 7.5, 500 mM NaCl, 25 mM imidazole). Protein was eluted with 25 ml IMAC elution  
5 buffer (50 mM sodium phosphate pH 7.5, 500 mM NaCl, 100 mM imidazole) and 4 ml fractions collected. The C6 antibody may be detected by absorbance at 280 nm and sFv fraction eluted. To remove dimeric and aggregated sFv, samples can be concentrated to a volume < 1 ml in a Centricon 10 (Amicon) and fractionated on a Superdex 75 column using a running buffer of HBS (10 mM Hepes, 150 mM NaCl, pH 7.4).

10 The purity of the final preparation may be evaluated by assaying an aliquot by SDS-PAGE. The protein bands can be detected by Coomassie staining. The concentration can then be determined spectrophotometrically, assuming that an  $A_{280}$  nm of 1.0 corresponds to an sFv concentration of 0.7 mg/ml .

## 15 II. Modification of C6 Antibodies.

### A) Display of antibody fragments on the surface of bacteriophage (phage display).

Display of antibody fragments on the surface of viruses which infect bacteria (bacteriophage or phage) makes it possible to produce human sFvs with a wide  
20 range of affinities and kinetic characteristics. To display antibody fragments on the surface of phage (phage display), an antibody fragment gene is inserted into the gene encoding a phage surface protein (pIII) and the antibody fragment-pIII fusion protein is expressed on the phage surface (McCafferty *et al.* (1990) *Nature*, 348: 552-554; Hoogenboom *et al.* (1991) *Nucleic Acids Res.*, 19: 4133-4137). For example, a sFv  
25 gene coding for the  $V_H$  and  $V_L$  domains of an anti-lysozyme antibody (DI.3) was inserted into the phage gene III resulting in the production of phage with the DI.3 sFv joined to the N-terminus of pIII thereby producing a "fusion" phage capable of binding lysozyme (McCafferty *et al.* (1990) *Nature*, 348: 552-554).

30 Since the antibody fragments on the surface of the phage are functional, phage bearing antigen binding antibody fragments can be separated from non-binding or lower affinity phage by antigen affinity chromatography (McCafferty *et al.* (1990) *Nature*, 348: 552-554). Mixtures of phage are allowed to bind to the affinity matrix, non-binding or lower affinity phage are removed by washing, and bound phage are eluted

by treatment with acid or alkali. Depending on the affinity of the antibody fragment, enrichment factors of 20 fold-1,000,000 fold are obtained by single round of affinity selection. By infecting bacteria with the eluted phage, however, more phage can be grown and subjected to another round of selection. In this way, an enrichment of 1000 fold in one round becomes 1,000,000 fold in two rounds of selection (McCafferty *et al.* (1990) *Nature*, 348: 552-554). Thus, even when enrichments in each round are low (Marks *et al.* (1991) *J. Mol. Biol.*, 222: 581-597), multiple rounds of affinity selection leads to the isolation of rare phage and the genetic material contained within which encodes the sequence of the binding antibody. The physical link between genotype and phenotype provided by phage display makes it possible to test every member of an antibody fragment library for binding to antigen, even with libraries as large as 100,000,000 clones. For example, after multiple rounds of selection on antigen, a binding sFv that occurred with a frequency of only 1/30,000,000 clones was recovered (Marks *et al.* (1991) *J. Mol. Biol.*, 222: 581-597).

Analysis of binding is simplified by including an amber codon between the antibody fragment gene and gene III. This makes it possible to easily switch between displayed and soluble antibody fragments simply by changing the host bacterial strain. When phage are grown in a supE suppresser strain of *E. coli*, the amber stop codon between the antibody gene and gene III is read as glutamine and the antibody fragment is displayed on the surface of the phage. When eluted phage are used to infect a non-supressor strain, the amber codon is read as a stop codon and soluble antibody is secreted from the bacteria into the periplasm and culture media (Hoogenboom *et al.* (1991) *Nucleic Acids Res.*, 19: 4133-4137). Binding of soluble sFv to antigen can be detected, *e.g.*, by ELISA using a murine IgG monoclonal antibody (*e.g.*, 9E10) which recognizes a C-terminal *myc* peptide tag on the sFv (Evan *et al.* (1985) *Mol. Cell Biol.*, 5: 3610-3616; Munro *et al.* (1986) *Cell*, 46: 291-300), *e.g.*, followed by incubation with polyclonal anti-mouse Fc conjugated to horseradish peroxidase.

#### **B) Phage display can be used to increase antibody affinity.**

To create higher affinity antibodies, mutant sFv gene repertoires, based on the sequence of a binding sFv, are created and expressed on the surface of phage. Higher affinity sFvs are selected on antigen as described above and in Examples 1 and 2. One approach for creating mutant sFv gene repertoires has been to replace either the V<sub>H</sub>

or  $V_L$  gene from a binding sFv with a repertoire of nonimmune  $V_H$  or  $V_L$  genes (chain shuffling) (Clackson *et al.* (1991) *Nature*, 352: 624-628). Such gene repertoires contain numerous variable genes derived from the same germline gene as the binding sFv, but with point mutations (Marks *et al.* (1992) *Bio/Technology*, 10: 779-783). Using light chain shuffling and phage display, the binding avidities of a human sFv antibody fragment can be dramatically increased. *See, e.g.,* Marks *et al.* *Bio/Technology*, 10: 779-785 (1992) in which the affinity of a human sFv antibody fragment which bound the hapten phenyloxazolone (phox) was increased from 300 nM to 15 nM (20 fold) (Marks *et al.* (1992) *Bio/Technology*, 10: 779-783).

#### **C) Isolation and characterization of C6.5, a human sFv which binds c-erbB-2.**

Isolation and characterization of C6.5 is described in detail in the Examples below. Human sFvs which bound to c-erbB-2 were isolated by selecting the nonimmune human sFv phage antibody library (described in Example 1) on c-erbB-2 extracellular domain peptide immobilized on polystyrene. After five rounds of selection, 45 of 96 clones analyzed (45/96) produced sFv which bound c-erbB-2 by ELISA. Restriction fragments analysis and DNA sequencing revealed the presence of two unique human sFvs, C4 and C6.5. Both of these sFvs bound only to c-erbB-2 and not to a panel of 10 irrelevant antigens. Cell binding assays, however, indicated that only C6.5 bound c-erbB-2 expressed on cells, and thus this sFv was selected for further characterization.

#### **D) Purification of C6.5.**

To facilitate purification, the C6.5 sFv gene was subcloned into the expression vector pUC119 Sfi-NotmycHis which results in the addition of the myc peptide tag followed by a hexahistidine tag at the C-terminal end of the sFv. The vector also encodes the pectate lyase leader sequence which directs expression of the sFv into the bacterial periplasm where the leader sequence is cleaved. This makes it possible to harvest native properly folded sFv directly from the bacterial periplasm. Native C6.5 sFv was expressed and purified from the bacterial supernatant using immobilized metal affinity chromatography. The yield after purification and gel filtration on a Superdex 75 column was 10.5 mg/L. Other C6 antibodies may be purified in a similar manner.

### E) Measurement of C6.5 affinity for c-erbB-2.

As explained above, selection for increased avidity involves measuring the affinity of a C6 antibody (e.g. a modified C6.5) for c-erbB-2. Methods of making such measurements are described in detail in Examples 1 and 2. Briefly, for example, the  $K_d$  of C6.5 and the kinetics of binding to c-erbB-2 were determined in a BIAcore, a biosensor based on surface plasmon resonance. For this technique, antigen is coupled to a derivatized sensor chip capable of detecting changes in mass. When antibody is passed over the sensor chip, antibody binds to the antigen resulting in an increase in mass which is quantifiable. Measurement of the rate of association as a function of antibody concentration can be used to calculate the association rate constant ( $k_{on}$ ). After the association phase, buffer is passed over the chip and the rate of dissociation of antibody ( $k_{off}$ ) determined. Rate constant  $k_{on}$  is typically measured in the range  $1.0 \times 10^2$  to  $5.0 \times 10^6$  and  $k_{off}$  in the range  $1.0 \times 10^{-1}$  to  $1.0 \times 10^{-6}$ . The equilibrium constant  $K_d$  is often calculated as  $k_{off}/k_{on}$  and thus is typically measured in the range  $10^{-5}$  to  $10^{-12}$ . Affinities measured in this manner correlate well with affinities measured in solution by fluorescence quench titration.

### F) Affinity of C6.5 for c-erbB-2.

The kinetics of binding and affinity of purified C6.5 were determined by BIAcore and the results are shown in Table 2. The  $K_d$  of  $1.6 \times 10^{-8}$  M determined by BIAcore is in close agreement to the  $K_d$  determined by Scatchard analysis after radioiodination ( $2.0 \times 10^{-8}$  M). C6.5 has a rapid  $k_{on}$ , and a relatively rapid  $k_{off}$ . The rapid  $k_{off}$  correlates with the *in vitro* measurement that only 22% of an injected dose is retained on the surface of SK-OV-3 cells after 30 minutes. Biodistribution of C6.5 was determined and the percent injected dose/gm tumor at 24 hours was 1.1% with tumor/organ ratios of 5.6 for kidney and 103 for bone. These values compare favorably to values obtained for 741F8 sFv. 741F8 is a monoclonal antibody capable of binding c-erbB-2 (see, e.g., U.S. Patent 5,169,774). The  $K_d$  of 741F8 was also measured by BIAcore and agreed with the value determined by scatchard analysis (Table 1).

**Table 1 Characterization of anti-c-erbB-2 sFv species.** Characteristics of the murine anti-c-erbB-2 sFv, 741F8, and the human sFv C6.5 are compared. The affinity and dissociation constants were determined by Scatchard plot analysis, unless otherwise stated. Dissociation from c-erbB-2 positive (SK-OV-3) cells was measured in an in vitro live cell assay. The percentage of injected dose per gram (%ID/g) tumor and tumor to organ ratios were determined in biodistribution studies performed in separate groups of scid mice (n=10-14) bearing SK-OV-3 tumors overexpressing c-erbB-2. SEM are < 35% of the associated values. a = significantly improved ( $p < 0.05$ ) compared to 741F8 sFv.

Parameter	741F8	C6.5
$K_d$ (BIAcore)	$2.6 \times 10^{-8}$ M	$1.6 \times 10^{-8}$ M
$K_d$ (Scatchard)	$5.4 \times 10^{-8}$ M	$2.1 \times 10^{-8}$ M
$k_{on}$ (BIAcore)	$2.4 \times 10^5$ M <sup>-1</sup> s <sup>-1</sup>	$4.0 \times 10^5$ M <sup>-1</sup> s <sup>-1</sup>
$k_{off}$ (BIAcore)	$6.4 \times 10^{-3}$ s <sup>-1</sup>	$6.3 \times 10^{-3}$ s <sup>-1</sup>
% associated with cell surface at 15 min	32.7%	60.6%
% associated with cell surface at 30 min	8.6%	22.2%
%ID/g Tumor	0.8	1.0
T:Blood	14.7	22.9
T:Kidney	2.8	5.6a
T:Liver	14.2	22.3
T:Spleen	10.3	34.1
T:Intestine	25.0	29.7
T:Lung	9.4	15.8
T:Stomach	8.9	11.1
T:Muscle	78.8	158.7
T:Bone	30.0	102.7

These results show that a human sFv which binds specifically to c-erbB-2 with moderate affinity was been produced. The sFv expresses at high level in *E. coli* as native sFv, and can be easily purified in high yield in two steps. Techniques are known for the rapid and efficiently purification of sFv from the bacterial periplasm and to measure affinity without the need for labeling.

**G) Estimating the affinity of unpurified sFv for c-erbB-2.**

Phage display and selection generally results in the selection of higher affinity mutant sFvs (Marks *et al.* (1992) *Bio/Technology*, 10: 779-783; Hawkins *et al.* (1992) *J. Mol. Biol.* 226: 889-896; Riechmann *et al.* (1993) *Biochemistry*, 32: 8848-8855; Clackson *et al.* (1991) *Nature*, 352: 624-628), but probably does not result in the separation of mutants with less than a 6 fold difference in affinity (Riechmann *et al.* (1993) *Biochemistry*, 32: 8848-8855). Thus a rapid method is needed to estimate the relative affinities of mutant sFvs isolated after selection. Since increased affinity results primarily from a reduction in the  $k_{\text{off}}$ , measurement of  $k_{\text{off}}$  should identify higher affinity sFv.  $k_{\text{off}}$  can be measured in the BLkcore on unpurified sFv in bacterial periplasm, since expression levels are high enough to give an adequate binding signal and  $k_{\text{off}}$  is independent of concentration. The value of  $k_{\text{off}}$  for periplasmic and purified sFv is in close agreement (Table 2).

Table 2. Comparison of  $k_{\text{off}}$  determined on sFv in bacterial periplasm and after purification by IMAC and gel filtration.

sFv	$k_{\text{off}}$ ( $\text{s}^{-1}$ )
C6-5 periplasm	$5.7 \times 10^{-3}$
C6-5 purified	$6.3 \times 10^{-3}$
C6-5ala3 periplasm	$9.3 \times 10^{-3}$
C6-5ala3 purified	$1.5 \times 10^{-3}$
C6-5ala10 periplasm	$3.7 \times 10^{-3}$
C6-5ala10 purified	$4.1 \times 10^{-3}$

Ranking of sFv by  $k_{\text{off}}$ , and hence relative affinity, can be determined without purification. Determination of relative affinity without purification significantly increases the rate at which mutant sFv are characterized, and reduces the number of mutant sFv subcloned and purified which do not show improved binding characteristics over C6.5 (*see* results of light chain shuffling and randomization below).

**H) Increasing the affinity of C6.5 by chain shuffling.**

To alter the affinity of C6.5, a mutant sFv gene repertoire was created containing the VH gene of C6.5 and a human VL gene repertoire (light chain shuffling).

The sFv gene repertoire was cloned into the phage display vector pHEN-1 (Hoogenboom *et al.* (1991) *Nucleic Acids Res.*, 19: 4133-4137) and after transformation a library of  $2 \times 10^5$  transformants was obtained. Phage were prepared and concentrated as described in Example 1 or 2.

5                    Selections were performed by incubating the phage with biotinylated c-erbB-2 in solution. The antigen concentration was decreased each round, reaching a concentration less than the desired  $K_d$  by the final rounds of selection. This results in the selection of phage on the basis of affinity (Hawkins *et al.* (1992) *J. Mol. Biol.* 226: 889-896). After four rounds of selection, 62/90 clones analyzed produced sFv which  
10 bound c-erbB-2 by ELISA. Single chain Fv was expressed from 48 ELISA positive clones (24 from the 3rd round of selection and 24 from the 4th round of selection), the periplasm harvested, and the sFv  $k_{off}$  determined by BIAcore. Single-chain Fvs were identified with a  $k_{off}$  three times slower than C6.5. The light chain gene of 10 of these sFvs was sequenced. One unique light chain was identified, C6L1. This sFv was  
15 subcloned into the hexahistidine vector, and expressed sFv purified by IMAC and gel filtration. Affinity was determined by BIAcore (Table 3).

Table 3. Affinity and kinetics of binding of C6.5 light and heavy chain shuffled mutant sFv.

20

sFv clone	$K_d$ (M)	$k_{on}$ ( $M^{-1} s^{-1}$ )	$k_{off}$ ( $s^{-1}$ )
C6.5	$1.6 \times 10^{-8}$	$4.0 \times 10^5$	$6.3 \times 10^{-3}$
C6L1 (light chain shuffle)	$2.6 \times 10^{-9}$	$7.8 \times 10^5$	$2.0 \times 10^{-3}$
C6VHB-4 (heavy chain shuffle)	$4.8 \times 10^{-9}$	$1.25 \times 10^6$	$6.0 \times 10^{-3}$
25 C6VHC (heavy chain shuffle)	$3.1 \times 10^{-9}$	$8.4 \times 10^5$	$2.6 \times 10^{-3}$

For heavy chain shuffling, the C6.5 VH CDR3 and light chain were cloned into a vector containing a human VH gene repertoire to create a phage antibody library of  $1 \times 10^6$  transformants. Selections were performed on biotinylated c-erbB-2 and  
30 after four rounds of selection, 82/90 clones analyzed produced sFv which bound c-erbB-2 by ELISA- sFv was expressed from 24 ELISA positive clones (24 from the 3rd round of selection and 24 from the 4th round of selection), the periplasm harvested, and the sFv  $k_{off}$  determined by BIAcore. Two clones were identified which had slower  $k_{off}$  than C6.5 (C6VHB-4 and C6VHC-4). Both of these were subcloned, purified, and affinities

determined by BIACore (Table 3). The affinity of C6.5 was increased 5 fold by heavy chain shuffling and 6 fold by light chain shuffling.

**D) Increasing the affinity of C6-5 by site directed mutagenesis of the third CDR of the light chain.**

5 The majority of antigen contacting amino acid side chains are located in the complementarity determining regions (CDRs), three in the  $V_H$  (CDR1, CDR2, and CDR3) and three in the  $V_L$  (CDR1, CDR2, and CDR3) (Chothia *et al.* (1987) *J. Mol. Biol.*, 196: 901-917; Chothia *et al.* (1986) *Science*, 233: 755-8; Nhan *et al.* (1991) *J. Mol. Biol.*, 217: 133-151). These residues contribute the majority of binding energetics responsible for antibody affinity for antigen. In other molecules, mutating amino acids which contact ligand has been shown to be an effective means of increasing the affinity of one protein molecule for its binding partner (Lowman *et al.* (1993) *J. Mol. Biol.*, 234: 564-578; Wells (1990) *Biochemistry*, 29: 8509-8516). Thus mutation (randomization) of the CDRs and screening against c-erbB-2 may be used to generate C6 antibodies having improved binding affinity.

15 For example, to increase the affinity of C6.5 for c-erbB-2, nine amino acid residues located in VL CDR3 (residues 89-95b, numbering according to Kabat *et al.* (1987) *supra.*; Table 2). were partially randomized by synthesizing a 'doped' oligonucleotide in which the wild type nucleotide occurred with a frequency of 49%. The oligonucleotide was used to amplify the remainder of the C6.5 sFv gene using PCR. The resulting sFv gene repertoire was cloned into pCANTAB5E (Pharmacia) to create a phage antibody library of  $1 \times 10^7$  transformants. The mutant phage antibody library was designated C6VLCDR3.

25 Selection of the C6.5 mutant VL CDR3 library (C6VLCDR3) was performed on biotinylated c-erbB-2 as described above for light chain shuffling. After three rounds of selection 82/92 clones analyzed produced sFv which bound c-erbB-2 by ELISA and after 4 rounds of selection, 92/92 clones analyzed produced sFv which bound c-erbB-2. Single-chain Fv was expressed from 24 ELISA positive clones from the 3rd and 4th rounds of selection, the periplasm harvested, and the  $k_{off}$  determined by BIACore. The best clones had a  $k_{off}$  approximately 5 to 10 times slower than that of C6.5. The light chain genes of 12 sFvs with the slowest  $k_{off}$  times from the 3rd and fourth round of selection were sequenced and each unique sFv subcloned into pUC119 Sfi-NotmycHis.

30

Single-chain Fv was expressed, purified by IMAC and gel filtration, and sFv affinity and binding kinetics determined by BIAcore (Table 4). Mutant sFv were identified with 16 fold increased affinity for c-erbB-2.

5 Table 4. Amino acid sequence, affinity, and binding kinetics of sFv isolated from a library of C6.5 mutants. Table identified mutants isolated after the third and fourth rounds of selection. The entire VL CDR3 of C6.5 is shown with the residues subjected to mutagenesis (89-95b) underlined. Rate constants  $k_{on}$  and  $k_{off}$  were measured on  
10 purified and gel filtered sFv by SPR in a BIAcore and the Kd calculated. A hyphen "-" indicates that there is no change from the C6.5  $V_L$  CDR3 sequence at that position.

sFv clone	$V_L$ CDR3 sequence	$k_d$ (M)	$k_{on}$ ( $M^{-1} s^{-1}$ )	$k_{off}$ ( $s^{-1}$ )
C6.5	8      9      9 9      5ab 7 <u>AAWDDSLSGWV</u>	$1.6 \times 10^{-8}$	$4.0 \times 10^5$	$6.3 \times 10^{-3}$
<b>3rd Round of selection:</b>				
C6ML3-5	----Y-----	$3.2 \times 10^{-9}$	$5.9 \times 10^5$	$1.9 \times 10^{-3}$
C6ML3-2	----H-----	$2.8 \times 10^{-9}$	$7.1 \times 10^5$	$2.0 \times 10^{-3}$
C6ML3-6	-S--Y-----	$3.2 \times 10^{-9}$	$5.9 \times 10^5$	$1.9 \times 10^{-3}$
C6ML3-1	----Y--W---	$6.7 \times 10^{-9}$	$3.0 \times 10^5$	$2.0 \times 10^{-3}$
C6ML3-3	-T--YA-----	$4.3 \times 10^{-9}$	$4.6 \times 10^5$	$2.0 \times 10^{-3}$
C6ML3-7	----YAV----	$2.6 \times 10^{-9}$	$6.5 \times 10^5$	$1.7 \times 10^{-3}$
C6ML3-4	-S-EY--W---	$3.5 \times 10^{-9}$	$4.0 \times 10^5$	$1.4 \times 10^{-3}$
<b>4th Round of selection:</b>				
C6ML3-12	----Y-R----	$1.6 \times 10^{-9}$	$4.5 \times 10^5$	$7.2 \times 10^{-4}$
C6ML3-9	-S--YT-----	$1.0 \times 10^{-9}$	$6.1 \times 10^5$	$9.2 \times 10^{-4}$
C6ML3-10	---E-PWY---	$2.3 \times 10^{-9}$	$6.1 \times 10^5$	$1.4 \times 10^{-3}$
C6ML3-11	----YA-W---	$3.6 \times 10^{-9}$	$6.1 \times 10^5$	$2.2 \times 10^{-3}$
C6ML3-13	----AT-W---	$2.4 \times 10^{-9}$	$8.7 \times 10^5$	$2.1 \times 10^{-3}$
C6ML3-8	----HLRW---	$2.6 \times 10^{-9}$	$6.5 \times 10^5$	$1.7 \times 10^{-3}$
C6ML3-23	-S--H--W---	$1.5 \times 10^{-9}$	$6.7 \times 10^5$	$1.7 \times 10^{-3}$
C6ML3-19	-S--RP-W---	$1.5 \times 10^{-9}$	$6.7 \times 10^5$	$1.0 \times 10^{-3}$
C6ML3-29	----GT-W---	$2.7 \times 10^{-9}$	$12.9 \times 10^5$	$2.2 \times 10^{-3}$
C6ML3-15	----RP-W---	$2.2 \times 10^{-9}$	$5.9 \times 10^5$	$1.3 \times 10^{-3}$
C6ML3-14	-----P-W---	$1.0 \times 10^{-9}$	$7.7 \times 10^5$	$7.7 \times 10^{-4}$

Partial randomization of a single CDR ( $V_L$  CDR3) resulted in the creation of mutant sFvs with 16 fold higher affinity for c-erbB-2, indicating that CDR randomization is an effective means of creating higher affinity sFv. The results also show that the method of selecting and identifying higher affinity sFv by reducing soluble antigen concentration during selections and screening periplasms by BIAcore prior to sequencing, subcloning and purification provides an effective way to isolate high affinity antibodies.

**D) Creation of C6.5 (sFv')<sub>2</sub> and (sFv)<sub>2</sub> homodimers and effect on affinity and binding kinetics for cerbB-2.**

To create C6 (sFv')<sub>2</sub> antibodies, two C6 sFvs are joined through a disulfide bond, or linker (e.g., a carbon linker) between the two cysteines. To create C6 (sFv)<sub>2</sub>, two C6 sFv are joined directly through a peptide bond or through a peptide linker. Thus, for example, to create disulfide linked C6.5 sFv', a cysteine residue was introduced by site directed mutagenesis between the myc tag and hexahistidine tag at the carboxy-terminus of C6.5. Introduction of the correct sequence was verified by DNA sequencing. The construct is in pUC119, the pelB leader directs expressed sFv' to the periplasm and cloning sites (NcoI and NotI) exist to introduce C6.5 mutant sFv'. This vector is called pUC119/C6.5 mycCysHis. Expressed sFv' has the myc tag at the C-terminus, followed by 2 glycines, a cysteine, and then 6 histidines to facilitate purification by IMAC. After disulfide bond formation between the two cysteine residues, the two sFv' are separated from each other by 26 amino acids (two 11 amino acid myc tags and 4 glycines). An sFv' was expressed from this construct, purified by IMAC, and analyzed by gel filtration. The majority of the sFv' was monomeric. To produce (sFv')<sub>2</sub> dimers, the cysteine was reduced by incubation with 1 MM beta-mercaptoethanol, and half of the sFv' blocked by the addition of DTNB. Blocked and unblocked sFv's were incubated together to form (sFv')<sub>2</sub> and the resulting material analyzed by gel filtration. 50% of the monomer was converted to (sFv')<sub>2</sub> homodimer as determined by gel filtration and nonreducing polyacrylamide gel electrophoresis. The affinity of the C6.5 sFv' monomer and (sFv')<sub>2</sub> dimer were determined by BIAcore (Table 5). The apparent affinity (avidity) of C6.5 increases 40 fold when converted to an (sFv')<sub>2</sub> homodimer.

Table 5. Affinities and binding kinetics of C6.5 sFv and C6.5 (sFv')<sub>2</sub>.

Clone	K <sub>d</sub> (M)	k <sub>on</sub> (M <sup>-1</sup> s <sup>-1</sup> )	k <sub>off</sub> (s <sup>-1</sup> )
C6.5 monomer	1.6 x 10 <sup>-8</sup>	4.0 x 10 <sup>5</sup>	6.3 x 10 <sup>-3</sup>
C6.5 dimer	4.0 x 10 <sup>-10</sup>	6.7 x 10 <sup>5</sup>	2.7 x 10 <sup>-4</sup>

The C6.5 (sFv')<sub>2</sub> exhibits a significant avidity effect compared to the sFv. Thus, this approach increases antibody fragment affinity, while remaining below the renal threshold for excretion.

In a particularly preferred embodiment, the (sFv)<sub>2</sub> dimer is expressed as a diabody (Holliger *et al.* (1993) *Proc. Natl. Acad. Sci. USA*, 90: 6444-6448; and WO 94/13804). This yields a bivalent molecule consisting of two C6.5 sFv polypeptide chains, since the VH and VL on the same peptide chain cannot pair. The production of a peptide linked C6.5 diabody is described in Example 5, below. In this example, the peptide linker sequence between the VH and VL domains was shortened from 15 amino acids to 5 amino acids. Synthetic oligonucleotides encoding the 5 amino acid linker (G<sub>4</sub>S) were used to PCR amplify the C6.5 V<sub>H</sub> and V<sub>L</sub> genes which were then spliced together to create the C6.5 diabody gene. The gene was then cloned into an appropriate vector, expressed, and purified according to standard methods well known to those of skill in the art. In another preferred embodiment, the (sFv)<sub>2</sub> dimer is produced using a longer peptide linker that permits the Vh and Vl to pair, yielding a single polypeptide chain with two C6 binding sites.

#### K) Effect of sFv affinity on *in vitro* cell binding and *in vivo* biodistribution.

As described in the preceding section, chain-shuffled and point-mutation variants of C6.5 have been prepared with K<sub>d</sub> ranging from 1.0 x 10<sup>-6</sup> M to 1.0 x 10<sup>-9</sup> M. The mutant sFv have been used to examine the effects of binding affinity and kinetics on *in vitro* cell binding and on *in vivo* biodistribution. Cell surface retention assays demonstrate that higher affinity sFv are retained to a much greater extent than lower affinity sFv. For sFv of approximately the same affinity, sFv with slower k<sub>off</sub> are better retained on the cell surface. In competitive binding assays, all of the molecules compete

in a dose dependent fashion with biotinylated C6.5 for c-erbB-2 on the surface of SK-BR-3 cells.

Twenty four hour biodistribution studies were performed in scid micebearing s.c. SK-OV-3 tumors to examine the role of affinity in the specificity and degree of tumorretention. These assays employed  $^{125}\text{I}$ -labeled forms of C6.5, C6G98A, C6ML3-9 and a negative control sFv at a dose of 25 mg. The c-erbB-2-specific sFv were selected to provide the following stepwise increase in affinity; C6G98A ( $3.2 \times 10^{-7}$ ), C6.5 ( $1.6 \times 10^{-8}$ ) and C6ML3-9 ( $1.0 \times 10^{-9}$ ). The biodistribution studies revealed a close correlation between the affinity and the %ID/g of the radioiodinated sFv retained in tumor. The greatest degree of tumor retention was observed with  $^{125}\text{I}$ -C6ML3-9 ( $1.42 \pm 0.23$  %ID/g). Significantly less tumor retention was achieved with  $^{125}\text{I}$ -C6.5 ( $0.80 \pm 0.07$  %ID/g) ( $p=0.0306$ ). Finally, the tumor retention of the lowest affinity clone  $^{125}\text{I}$ -C6G98A ( $0.19 \pm 0.04$  %ID/g) was significantly less than that of C6.5 ( $p=0.00001$ ) and was identical to that of the negative control  $^{125}\text{I}$ -26-10. The T:O ratios also reflected the greater retention of higher-affinity species in tumor. For example, tumor:blood ratios of 17.2, 13.3, 3.5 and 2.6, and tumor to liver ratios of 26.2, 19.8, 4.0 and 3.1 were observed for C6ML3-9, C6.5, C6G98A and 26-10, respectively.

These results demonstrate that selective tumor retention of sFv molecules correlates with their affinity properties. With further increases in affinity, additional improvements in tumor retention are observed.

#### L) Approach to produce higher affinity human sFv.

As described above and in Examples 1 and 2, a C6 antibody (e.g. C6.5 sFv), which binds specifically to c-erbB-2, is expressed at high level in *E. coli* as native protein, and can be simply purified in high yield. Optimized techniques for creating large C6.5 mutant phage antibody libraries and developed techniques for efficiently selecting higher affinity mutants from these libraries are provided. These techniques were used to increase C6.5 affinity 16 fold, to  $1.0 \times 10^{-9}$  M, by randomizing  $V_L$  CDR3, and 5 and 6 fold by heavy and light chain shuffling respectively.

To further increase affinity, mutant C6.5 phage antibody libraries can be created where the other CDRs are randomized ( $V_L$  CDR1 and CDR2 and  $V_H$  CDR1, CDR2 and CDR3). Each CDR is randomized in a separate library, using, for example, C6ML3-9 as a template ( $K_d = 1.0 \times 10^{-9}$  M). In a preferred embodiment, CDRs can be

sequentially randomized, using the highest affinity sFv as the template for the next round of mutagenesis. This approach would be preferred when mutating CDRs that pack on each other, for example VL and VH CDR3. In another embodiment, CDRs could be mutated in parallel, and mutations combined to achieve an additive effect on affinity.

5 This approach has been used to increase the affinity of human growth hormone (hGH) for the growth hormone receptor over 1500 fold from  $3.4 \times 10^{-10}$  to  $9.0 \times 10^{-13}$  M (Lowman *et al.* (1993) *J. Mol. Biol.*, 234: 564-578).

$V_H$  CDR3 occupies the center of the binding pocket, and thus mutations in this region are likely to result in an increase in affinity (Clackson *et al.* (1995) *Science*, 10 267: 383-386). In one embodiment, four  $V_H$  CDR3 residues at a time are randomized using the nucleotides NNS. To create the library, an oligonucleotide is synthesized which anneals to the C6.5  $V_H$  framework 3 and encodes  $V_H$  CDR3 and a portion of framework 4. At the four positions to be randomized, the sequence NNS is used, where N = any of the 4 nucleotides, and S = C or T. The oligonucleotide are used to amplify 15 the C6.5  $V_H$  gene using PCR, creating a mutant C6.5 VH gene repertoire. PCR is used to splice the VH gene repertoire with the C6ML3-9 light chain gene, and the resulting sFv gene repertoire cloned into the phage display vector pHEN-1. Ligated vector DNA is used to transform electrocompetent *E. coli* to produce a phage antibody library of >  $1.0 \times 10^7$  clones.

20 To select higher affinity mutant sFv, each round of selection of the phage antibody libraries is conducted on decreasing amounts of biotinylated c-erbB-2, as described in the Examples. Typically, 96 clones from the third and fourth round of selection are screened for binding to c-erbB-2 by ELISA on 96 well plates. Single-chain Fv from twenty to forty ELISA positive clones are expressed in 10 ml cultures, the 25 periplasm harvested, and the sFv  $k_{off}$  determined by BIAcore. Clones with the slowest  $k_{off}$  are sequenced, and each unique sFv subcloned into pUC119 SfiNotmycHis. Single chain Fv is expressed in 1L cultures, and purified as described *supra*. Affinities of purified sFv are determined by BIAcore. Randomization of one four amino acid segment of  $V_H$  CDR3 produces a C6 mutant with a  $K_D$  of  $1.6 \times 10^{-10}$  M (see Example 3).

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**M) In vitro cell binding assays, in vivo pharmacokinetic and biodistribution studies.**

Once higher affinity sFv's are identified, production is scaled up to provide adequate material for *in vitro* cell binding assays and *in vivo* pharmacokinetic and biodistribution studies. Techniques for scaling up production are known. Briefly, in one embodiment, sFv is expressed in *E. coli* cultures grown in 2 liter shaker flasks. Single-chain Fv is purified from the periplasm as described above and in Examples 1 and 2. Mutant sFv of higher affinity are tested using the cell retention assay described in Examples 1 and 2. Since the  $t_{1/2}$  of retention should be approximately two hours when  $k_{off}$  is less than  $10^{-4}$ , the assay is done at 30, 60, 120, 240 minutes and 18 hour incubations. Scatchard analyses may be performed on selected samples.

These studies show that affinities measured in the BIAcore on immobilized antigen correspond to improved cell binding. The pharmacokinetic and biodistribution properties of sFv molecules with broadly different affinity characteristics are screened using labeled sFv and scid mice bearing human SK-OV-3 tumors. This serves to identify molecules with *in vivo* properties that make them unsuitable for use as therapeutics *i.e.*, unexpected aggregation, or unacceptable normal organ retention properties.

Twenty four hour biodistribution results are convenient indicators of overall biodistribution properties. C6 antibodies, for example C6.5 mutants, with affinities between  $1.6 \times 10^{-8}$  M and  $1.0 \times 10^{-11}$  M, and which differ at least 3 to 4 fold in affinity, are screened. Mutants with similar  $K_d$  but with dissimilar  $k_{off}$  are also studied. A number of C6.5 series affinity variants are tested and more extensive biodistribution studies performed on molecules that differ significantly from C6.5 or the nearest affinity variant in 24 hour biodistribution characteristics. These data are used to generate tissue-specific AUC determinations, as well as tumor:normal organ AUC ratios and MIRD estimates.

Sample molecules associated with favorable predicted human dosimetry (*e.g.*, based upon the MIRD formulation) are assayed for their *in vivo* therapeutic efficacy in mice.

An affinity of  $1.0 \times 10^{-11}$  can be chosen as an endpoint in this preferred embodiment because the associated  $k_{off}$  ( $10^{-5}$ ) results in a  $t_{1/2}$  for dissociation from tumor of greater than 20 hours. Higher affinity endpoints can be selected and result in even longer retention. The  $t_{1/2}$  for dissociation of C6.5 is approximately 3 minutes. This

invention provides optimized techniques for creating large C6.5 mutant phage antibody libraries and techniques for efficiently selecting higher affinity mutants from these libraries. A number of C6.5 mutants with affinities between  $1.6 \times 10^{-8}$  M to  $1.0 \times 10^{-10}$  M are provided. Combining these mutations into the same sFv produces sFv mutants with  $K_d$  between  $1.6 \times 10^{-10}$  M and  $3.3 \times 10^{-11}$  M.

**N) Preparation of C6 (sFv)<sub>2</sub>, (sFv')<sub>2</sub>, Fab, and (Fab')<sub>2</sub>, conjugates and diabodies.**

C6 antibodies such as C6.5 sFv, or a variant with higher affinity, are suitable templates for creating size and valency variants. For example, a C6.5 (sFv')<sub>2</sub> is created from the parent sFv as described above and in Example 1. An sFv' can be created by excising the sFv gene, e.g., with NcoI and NotI from pHEN-1 or pUC119 Sfi-NotmycHis and cloned into pUC119C6.5mycCysHis, cut with NcoI and NotI. In one embodiment, expressed sFv' has a myc tag at the C-terminus, followed by 2 glycines, a cysteine, and 6 histidines to facilitate purification. After disulfide bond formation between the two cysteine residues, the two sFv should be separated from each other by 26 amino acids (e.g., two 11 amino acid myc tags and 4 glycines). sFv is expressed from this construct and purified. To produce (sFv')<sub>2</sub> dimers, the cysteine is reduced by incubation with 1 Mm beta-mercaptoethanol, and half of the sFv blocked by the addition of DTNB. Blocked and unblocked sFv are incubated together to form (sFv')<sub>2</sub>, which is purified. This approach was used to produce C6.5 (sFv')<sub>2</sub> dimer, which demonstrates a 40 fold higher affinity than C6.5. A (sFv')<sub>2</sub> may be constructed for example, from C6L1 ( $K_d = 2.5 \times 10^{-9}$  M) and C6ML3-9 ( $K_d = 1.0 \times 10^{-9}$  M). As higher affinity sFv become available, their genes are similarly used to construct (sFv')<sub>2</sub>.

Alternatively, C6 (sFv)<sub>2</sub> can be produced by linking the two sFv by a peptide, as described in Example 5. As higher affinity sFv become available their genes can be used to construct higher affinity (sFv)<sub>2</sub>.

C6.5 based Fab are expressed in *E. coli* using an expression vector similar to the one described by Better *et. al.* (Better *et al.* (1988) *Science*, 240: 1041-1043). To create a C6.5 based Fab, the VH and VL genes are amplified from the sFv using PCR. The VH gene is cloned into a PUC119 based bacterial expression vector which provides the human IgG CH1 domain downstream from, and in frame with, the V<sub>H</sub> gene. The vector also contains the lac promoter, a pelb leader sequence to direct expressed V<sub>H</sub>-CH1

domain into the periplasm, a gene 3 leader sequence to direct expressed light chain into the periplasm, and cloning sites for the light chain gene. Clones containing the correct VH gene are identified, e.g., by PCR fingerprinting. The VL gene is spliced to the CL gene using PCR and cloned into the vector containing the VH CH1 gene.

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### III. Preparation of Chimeric Molecules.

In another embodiment this invention provides for chimeric molecules comprising a C6 antibody attached to an effector molecule. As explained above, the effector molecule component of the chimeric molecules of this invention may be any molecule whose activity it is desired to deliver to cells that express c-erbB-2. Suitable effector molecules include cytotoxins such as PE, Ricin, Abrin or DT, radionuclides, ligands such as growth factors, antibodies, detectable labels such as fluorescent or radioactive labels, and therapeutic compositions such as liposomes and various drugs.

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#### A) Cytotoxins.

Particularly preferred cytotoxins include *Pseudomonas* exotoxins, *Diphtheria* toxins, ricin, and abrin. *Pseudomonas* exotoxin and *Diphtheria* toxin, in particular, are frequently used in chimeric cytotoxins.

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##### i) *Pseudomonas* exotoxin (PE).

*Pseudomonas* exotoxin A (PE) is an extremely active monomeric protein (molecular weight 66 kD), secreted by *Pseudomonas aeruginosa*, which inhibits protein synthesis in eukaryotic cells through the inactivation of elongation factor 2 (EF-2) by catalyzing its ADP-ribosylation (catalyzing the transfer of the ADP ribosyl moiety of oxidized NAD onto EF-2).

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The toxin contains three structural domains that act in concert to cause cytotoxicity. Domain Ia (amino acids 1-252) mediates cell binding. Domain II (amino acids 253-364) is responsible for translocation into the cytosol and domain III (amino acids 400-613) mediates ADP ribosylation of elongation factor 2, which inactivates the protein and causes cell death. The function of domain Ib (amino acids 365-399) remains undefined, although a large part of it, amino acids 365-380, can be deleted without loss of cytotoxicity. See Siegall *et al.*, *J. Biol. Chem.* 264: 14256-14261 (1989).

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For maximum cytotoxic properties of a preferred PE molecule, several modifications to the molecule are recommended. An appropriate carboxyl terminal sequence to the recombinant molecule is preferred to translocate the molecule into the cytosol of target cells. Amino acid sequences which have been found to be effective include, REDLK (as in native PE), REDL, RDEL, or KDEL, repeats of those, or other sequences that function to maintain or recycle proteins into the endoplasmic reticulum, referred to here as "endoplasmic retention sequences". See, for example, Chaudhary *et al.*, *Proc. Natl. Acad. Sci. USA* 87:308-312 and Seetharam *et al.*, *J. Biol. Chem.* 266: 17376-17381 (1991).

10 The targeting molecule can be inserted in replacement for domain Ia. A similar insertion has been accomplished in what is known as the TGF $\alpha$ -PE40 molecule (also referred to as TP40) described in Heimbrook *et al.*, *Proc. Natl. Acad. Sci., USA*, 87: 4697-4701 (1990). See also, Debinski *et al.* *Bioconj. Chem.*, 5: 40 (1994) for other PE variants).

15 The PE molecules can be fused to the C6 antibody by recombinant means. The genes encoding protein chains may be cloned in cDNA or in genomic form by any cloning procedure known to those skilled in the art. See for example Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, (1989). Methods of cloning genes encoding PE fused to various ligands are well known to those of skill in the art. See, for example, Siegall *et al.*, *FASEB J.*, 3: 2647-2652 (1989); Chaudhary *et al.* *Proc. Natl. Acad. Sci. USA*, 84: 4538-4542 (1987).

20 Those skilled in the art will realize that additional modifications, deletions, insertions and the like may be made to the chimeric molecules of the present invention or to the nucleic acid sequences encoding the C6 chimeric molecules. Especially, deletions or changes may be made in PE or in a linker connecting an antibody gene to PE, in order to increase cytotoxicity of the fusion protein toward target cells or to decrease nonspecific cytotoxicity toward cells without antigen for the antibody. All such constructions may be made by methods of genetic engineering well known to those skilled in the art (see, generally, Sambrook *et al.*, *supra*) and may produce proteins that have differing properties of affinity, specificity, stability and toxicity that make them particularly suitable for various clinical or biological applications.

**ii) Diphtheria toxin (DT).**

Like PE, diphtheria toxin (DT) kills cells by ADP-ribosylating elongation factor 2 (EF-2) thereby inhibiting protein synthesis. Diphtheria toxin, however, is divided into two chains, A and B, linked by a disulfide bridge. In contrast to PE, chain B of DT, which is on the carboxyl end, is responsible for receptor binding and chain A, which is present on the amino end, contains the enzymatic activity (Uchida *et al.*, *Science*, 175: 901-903 (1972); Uchida *et al. J. Biol. Chem.*, 248: 3838-3844 (1973)).

The targeting molecule-Diphtheria toxin fusion proteins of this invention may have the native receptor-binding domain removed by truncation of the Diphtheria toxin B chain. DT388, a DT in which the carboxyl terminal sequence beginning at residue 389 is removed is illustrated in Chaudhary, *et al.*, *Bioch. Biophys. Res. Comm.*, 180: 545-551 (1991).

Like the PE chimeric cytotoxins, the DT molecules may be chemically conjugated to the C6 antibody but, may also be prepared as fusion proteins by recombinant means. The genes encoding protein chains may be cloned in cDNA or in genomic form by any cloning procedure known to those skilled in the art. Methods of cloning genes encoding DT fused to various ligands are also well known to those of skill in the art. See, for example, Williams *et al. J. Biol. Chem.* 265: 11885-11889 (1990) which describes the expression of growth-factor-DT fusion proteins.

The term "Diphtheria toxin" (DT) as used herein refers to full length native DT or to a DT that has been modified. Modifications typically include removal of the targeting domain in the B chain and, more specifically, involve truncations of the carboxyl region of the B chain.

**B) Detectable labels.**

Detectable labels suitable for use as the effector molecule component of the chimeric molecules of this invention include any composition detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical or chemical means. Useful labels in the present invention include magnetic beads (*e.g.* Dynabeads™), fluorescent dyes (*e.g.*, fluorescein isothiocyanate, texas red, rhodamine, green fluorescent protein, and the like), radiolabels (*e.g.*, <sup>3</sup>H, <sup>125</sup>I, <sup>35</sup>S, <sup>14</sup>C, or <sup>32</sup>P), enzymes (*e.g.*, horse radish peroxidase, alkaline phosphatase and others commonly used in an ELISA), and colorimetric labels such as colloidal gold or colored glass or plastic

(*e.g.* polystyrene, polypropylene, latex, *etc.*) beads. Patents teaching the use of such labels include U.S. Patent Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241.

Means of detecting such labels are well known to those of skill in the art. Thus, for example, radiolabels may be detected using photographic film or scintillation counters, fluorescent markers may be detected using a photodetector to detect emitted illumination. Enzymatic labels are typically detected by providing the enzyme with a substrate and detecting the reaction product produced by the action of the enzyme on the substrate, and colorimetric labels are detected by simply visualizing the colored label.

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### C) Ligands.

As explained above, the effector molecule may also be a ligand or an antibody. Particularly preferred ligand and antibodies are those that bind to surface markers of immune cells. Chimeric molecules utilizing such antibodies as effector molecules act as bifunctional linkers establishing an association between the immune cells bearing binding partner for the ligand or antibody and the tumor cells expressing the c-erbB-2. Suitable antibodies and growth factors are known to those of skill in the art and include, but are not limited to, IL-2, IL-4, IL-6, IL-7, tumor necrosis factor (TNF), anti-Tac, TGF $\alpha$ , and the like.

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### D) Other therapeutic moieties.

Other suitable effector molecules include pharmacological agents or encapsulation systems containing various pharmacological agents. Thus, the C6 antibody may be attached directly to a drug that is to be delivered directly to the tumor. Such drugs are well known to those of skill in the art and include, but are not limited to, doxorubicin, vinblastine, genistein, antisense molecules, ribozymes and the like.

Alternatively, the effector molecule may comprise an encapsulation system, such as a liposome or micelle that contains a therapeutic composition such as a drug, a nucleic acid (*e.g.* an antisense nucleic acid), or another therapeutic moiety that is preferably shielded from direct exposure to the circulatory system. Means of preparing liposomes attached to antibodies are well known to those of skill in the art. See, for example, U.S. Patent No. 4,957,735 and Connor *et al.*, *Pharm. Ther.*, 28: 341-365 (1985).

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**E) Attachment of the c6 antibody to the effector molecule.**

One of skill will appreciate that the C6 antibody and the effector molecule may be joined together in any order. Thus the effector molecule may be joined to either the amino or carboxy termini of the C6 antibody. The C6 antibody may also be joined to an internal region of the effector molecule, or conversely, the effector molecule may be joined to an internal location of the C6 antibody as long as the attachment does not interfere with the respective activities of the molecules.

The C6 antibody and the effector molecule may be attached by any of a number of means well known to those of skill in the art. Typically the effector molecule is conjugated, either directly or through a linker (spacer), to the C6 antibody. However, where the effector molecule is a polypeptide it is preferable to recombinantly express the chimeric molecule as a single-chain fusion protein.

**i) Conjugation of the effector molecule to the targeting molecule.**

In one embodiment, the targeting molecule C6 antibody is chemically conjugated to the effector molecule (*e.g.* a cytotoxin, a label, a ligand, or a drug or liposome). Means of chemically conjugating molecules are well known to those of skill (see, for example, Chapter 4 in *Monoclonal Antibodies: Principles and Applications*, Birch and Lennox, eds. John Wiley & Sons, Inc. N.Y. (1995) which describes conjugation of antibodies to anticancer drugs, labels including radio labels, enzymes, and the like).

The procedure for attaching an agent to an antibody or other polypeptide targeting molecule will vary according to the chemical structure of the agent. Polypeptides typically contain variety of functional groups; *e.g.*, carboxylic acid (COOH) or free amine (-NH<sub>2</sub>) groups, which are available for reaction with a suitable functional group on an effector molecule to bind the effector thereto.

Alternatively, the targeting molecule and/or effector molecule may be derivatized to expose or attach additional reactive functional groups. The derivatization may involve attachment of any of a number of linker molecules such as those available from Pierce Chemical Company, Rockford Illinois.

A "linker", as used herein, is a molecule that is used to join the targeting molecule to the effector molecule. The linker is capable of forming covalent bonds to both the targeting molecule and to the effector molecule. Suitable linkers are well known

to those of skill in the art and include, but are not limited to, straight or branched-chain carbon linkers, heterocyclic carbon linkers, or peptide linkers. Where the targeting molecule and the effector molecule are polypeptides, the linkers may be joined to the constituent amino acids through their side groups (e.g., through a disulfide linkage to cysteine). However, in a preferred embodiment, the linkers will be joined to the alpha carbon amino and carboxyl groups of the terminal amino acids.

A bifunctional linker having one functional group reactive with a group on a particular agent, and another group reactive with an antibody, may be used to form the desired immunoconjugate. Alternatively, derivatization may involve chemical treatment of the targeting molecule, e.g., glycol cleavage of a sugar moiety attached to the protein antibody with periodate to generate free aldehyde groups. The free aldehyde groups on the antibody may be reacted with free amine or hydrazine groups on an agent to bind the agent thereto. (See U.S. Patent No. 4,671,958). Procedures for generation of free sulfhydryl groups on polypeptide, such as antibodies or antibody fragments, are also known (See U.S. Pat. No. 4,659,839).

Many procedure and linker molecules for attachment of various compounds including radionuclide metal chelates, toxins and drugs to proteins such as antibodies are known. See, for example, European Patent Application No. 188,256; U.S. Patent Nos. 4,671,958, 4,659,839, 4,414,148, 4,699,784; 4,680,338; 4,569,789; and 4,589,071; and Borlinghaus *et al.* *Cancer Res.* 47: 4071-4075 (1987) which are incorporated herein by reference. In particular, production of various immunotoxins is well-known within the art and can be found, for example in "Monoclonal Antibody-Toxin Conjugates: Aiming the Magic Bullet," Thorpe *et al.*, *Monoclonal Antibodies in Clinical Medicine*, Academic Press, pp. 168-190 (1982), Waldmann, *Science*, 252: 1657 (1991), U.S. Patent Nos. 4,545,985 and 4,894,443.

In some circumstances, it is desirable to free the effector molecule from the targeting molecule when the chimeric molecule has reached its target site. Therefore, chimeric conjugates comprising linkages which are cleavable in the vicinity of the target site may be used when the effector is to be released at the target site. Cleaving of the linkage to release the agent from the antibody may be prompted by enzymatic activity or conditions to which the immunoconjugate is subjected either inside the target cell or in the vicinity of the target site. When the target site is a tumor, a linker which is

cleavable under conditions present at the tumor site (*e.g.* when exposed to tumor-associated enzymes or acidic pH) may be used.

A number of different cleavable linkers are known to those of skill in the art. See U.S. Pat. Nos. 4,618,492; 4,542,225, and 4,625,014. The mechanisms for  
5 release of an agent from these linker groups include, for example, irradiation of a photolabile bond and acid-catalyzed hydrolysis. U.S. Pat. No. 4,671,958, for example, includes a description of immunoconjugates comprising linkers which are cleaved at the target site *in vivo* by the proteolytic enzymes of the patient's complement system. In  
10 view of the large number of methods that have been reported for attaching a variety of radiodiagnostic compounds, radiotherapeutic compounds, drugs, toxins, and other agents to antibodies one skilled in the art will be able to determine a suitable method for attaching a given agent to an antibody or other polypeptide.

#### ii) Production of fusion proteins.

15 Where the C6 antibody and/or the effector molecule are relatively short (*i.e.*, less than about 50 amino acids) they may be synthesized using standard chemical peptide synthesis techniques. Where both molecules are relatively short the chimeric molecule may be synthesized as a single contiguous polypeptide. Alternatively the C6  
20 antibody and the effector molecule may be synthesized separately and then fused by condensation of the amino terminus of one molecule with the carboxyl terminus of the other molecule thereby forming a peptide bond. Alternatively, the targeting and effector molecules may each be condensed with one end of a peptide spacer molecule thereby forming a contiguous fusion protein.

25 Solid phase synthesis in which the C-terminal amino acid of the sequence is attached to an insoluble support followed by sequential addition of the remaining amino acids in the sequence is the preferred method for the chemical synthesis of the polypeptides of this invention. Techniques for solid phase synthesis are described by Barany and Merrifield, *Solid-Phase Peptide Synthesis*; pp. 3-284 in *The Peptides: Analysis, Synthesis, Biology. Vol. 2: Special Methods in Peptide Synthesis, Part A.*,  
30 Merrifield, *et al. J. Am. Chem. Soc.*, 85: 2149-2156 (1963), and Stewart *et al.*, *Solid Phase Peptide Synthesis, 2nd ed.* Pierce Chem. Co., Rockford, Ill. (1984).

In a preferred embodiment, the chimeric fusion proteins of the present invention are synthesized using recombinant DNA methodology. Generally this involves

creating a DNA sequence that encodes the fusion protein, placing the DNA in an expression cassette under the control of a particular promoter, expressing the protein in a host, isolating the expressed protein and, if required, renaturing the protein.

5 DNA encoding the fusion proteins (*e.g.* C6.5Ab-PE) of this invention may be prepared by any suitable method, including, for example, cloning and restriction of appropriate sequences or direct chemical synthesis by methods such as the phosphotriester method of Narang *et al.* *Meth. Enzymol.* 68: 90-99 (1979); the phosphodiester method of Brown *et al.*, *Meth. Enzymol.* 68: 109-151 (1979); the diethylphosphoramidite method of Beaucage *et al.*, *Tetra. Lett.*, 22: 1859-1862 (1981);  
10 and the solid support method of U.S. Patent No. 4,458,066.

Chemical synthesis produces a single stranded oligonucleotide. This may be converted into double stranded DNA by hybridization with a complementary sequence, or by polymerization with a DNA polymerase using the single strand as a template. One of skill would recognize that while chemical synthesis of DNA is limited  
15 to sequences of about 100 bases, longer sequences may be obtained by the ligation of shorter sequences.

Alternatively, subsequences may be cloned and the appropriate subsequences cleaved using appropriate restriction enzymes. The fragments may then be ligated to produce the desired DNA sequence.

20 In a preferred embodiment, DNA encoding fusion proteins of the present invention may be cloned using DNA amplification methods such as polymerase chain reaction (PCR). Thus, for example, the gene for the C6 antibody may be amplified from a nucleic acid template (clone) using a sense primer containing a first restriction site and an antisense primer containing a second restriction site. This produces a nucleic acid  
25 encoding the mature C6 antibody sequence and having terminal restriction sites. A cytotoxin (or other polypeptide effector) may be cut out of a plasmid encoding that effector using restriction enzymes to produce cut ends suitable for annealing to the C6 antibody. Ligation of the sequences and introduction of the construct into a vector produces a vector encoding the C6-effector molecule fusion protein. Such PCR cloning  
30 methods are well known to those of skill in the art (*see, for example, Debinski et al. Int. J. Cancer*, 58: 744-748 (1994), for an example of the preparation of a PE fusion protein).

While the two molecules may be directly joined together, one of skill will appreciate that the molecules may be separated by a peptide spacer consisting of one or more amino acids. Generally the spacer will have no specific biological activity other than to join the proteins or to preserve some minimum distance or other spatial relationship between them. However, the constituent amino acids of the spacer may be selected to influence some property of the molecule such as the folding, net charge, or hydrophobicity. One of skill will appreciate that PCR primers may be selected to introduce an amino acid linker or spacer between the C6 antibody and the effector molecule if desired.

10           The nucleic acid sequences encoding the fusion proteins may be expressed in a variety of host cells, including *E. coli*, other bacterial hosts, yeast, and various higher eukaryotic cells such as the COS, CHO and HeLa cells lines and myeloma cell lines. The recombinant protein gene will be operably linked to appropriate expression control sequences for each host. For *E. coli* this includes a promoter such as the T7, 15 trp, or lambda promoters, a ribosome binding site and preferably a transcription termination signal. For eukaryotic cells, the control sequences will include a promoter and preferably an enhancer derived from immunoglobulin genes, SV40, cytomegalovirus, etc., and a polyadenylation sequence, and may include splice donor and acceptor sequences.

20           The plasmids of the invention can be transferred into the chosen host cell by well-known methods such as calcium chloride transformation for *E. coli* and calcium phosphate treatment or electroporation for mammalian cells. Cells transformed by the plasmids can be selected by resistance to antibiotics conferred by genes contained on the plasmids, such as the amp, gpt, neo and hyg genes.

25           Once expressed, the recombinant fusion proteins can be purified according to standard procedures of the art, including ammonium sulfate precipitation, affinity columns, column chromatography, gel electrophoresis and the like (*see, generally, R. Scopes, Protein Purification, Springer-Verlag, N.Y. (1982), Deutscher, Methods in Enzymology Vol. 182: Guide to Protein Purification., Academic Press, Inc. N.Y. (1990)*). In a preferred embodiment, the fusion proteins are purified using affinity 30 purification methods as described in Examples 1 and 2. Substantially pure compositions of at least about 90 to 95% homogeneity are preferred, and 98 to 99% or more

homogeneity are most preferred for pharmaceutical uses. Once purified, partially or to homogeneity as desired, the polypeptides may then be used therapeutically.

One of skill in the art would recognize that after chemical synthesis, biological expression, or purification, the C6 antibody-effector fusion protein may possess a conformation substantially different than the native conformations of the constituent polypeptides. In this case, it may be necessary to denature and reduce the polypeptide and then to cause the polypeptide to re-fold into the preferred conformation. Methods of reducing and denaturing proteins and inducing re-folding are well known to those of skill in the art. (See, Debinski *et al. J. Biol. Chem.*, 268: 14065-14070 (1993); Kreitman and Pastan, *Bioconjug. Chem.*, 4: 581-585 (1993); and Buchner, *et al., Anal. Biochem.*, 205: 263-270 (1992). Debinski *et al.*, for example, describe the denaturation and reduction of inclusion body proteins in guanidine-DTE. The protein is then refolded in a redox buffer containing oxidized glutathione and L-arginine.

One of skill would recognize that modifications can be made to the C6 antibody-effector fusion proteins without diminishing their biological activity. Some modifications may be made to facilitate the cloning, expression, or incorporation of the targeting molecule into a fusion protein. Such modifications are well known to those of skill in the art and include, for example, a methionine added at the amino terminus to provide an initiation site, or additional amino acids placed on either terminus to create conveniently located restriction sites or termination codons.

#### IV. Diagnostic Assays.

As explained above, the C6 antibodies may be used for the *in vivo* or *in vitro* detection of c-erbB-2 and thus, in the diagnosis and/or localization of cancers characterized by the expression of c-erbB-2.

##### A) *In Vivo* Detection of c-erbB-2.

The C6 antibodies and/or chimeric molecules of the present invention may be used for *in vivo* detection and localization of cells (*e.g.* c-erbB-2 positive carcinoma) bearing c-erbB-2. Such detection involves administering to an organism a chimeric molecule comprising a C6 joined to a label detectable *in vivo*. Such labels are well known to those of skill in the art and include, but are not limited to, electron dense labels such as gold or barium which may be detected by X-ray or CAT scan, various

radioactive labels that may be detected using scintillography, and various magnetic and paramagnetic materials that may be detected using positron emission tomography (PET) and magnetic resonance imaging (MRI). The C6 antibody associates the label with the c-erbB-2 bearing cell which is then detected and localized using the appropriate detection method.

**B) *In Vitro* Detection of c-erbB-2.**

The C6 antibodies of this invention are also useful for the detection of c-erbB-2 *in vitro e.g.*, in biological samples obtained from an organism. The detection and/or quantification of c-erbB-2 in such a sample is indicative the presence or absence or quantity of cells (*e.g.*, tumor cells) overexpressing c-erbB-2.

The c-erbB-2 antigen may be quantified in a biological sample derived from a patient such as a cell, or a tissue sample derived from a patient. As used herein, a biological sample is a sample of biological tissue or fluid that contains a c-erbB-2 antigen concentration that may be correlated with and indicative of cells overexpressing c-erbB-2. Preferred biological samples include blood, urine, and tissue biopsies.

In a particularly preferred embodiment, erbB-2 is quantified in breast tissue cells derived from normal or malignant breast tissue samples. Although the sample is typically taken from a human patient, the assays can be used to detect erbB-2 in cells from mammals in general, such as dogs, cats, sheep, cattle and pigs, and most particularly primates such as humans, chimpanzees, gorillas, macaques, and baboons, and rodents such as mice, rats, and guinea pigs.

Tissue or fluid samples are isolated from a patient according to standard methods well known to those of skill in the art, most typically by biopsy or venipuncture. The sample is optionally pretreated as necessary by dilution in an appropriate buffer solution or concentrated, if desired. Any of a number of standard aqueous buffer solutions, employing one of a variety of buffers, such as phosphate, Tris, or the like, at physiological pH can be used.

**C) Assay Formats (Detection or Quantification of c-erbB-2).**

**D) Immunological Binding Assays**

The c-erbB-2 peptide (analyte) or an anti-c-erbB-2 antibody is preferably detected in an immunoassay utilizing a C6 antibody as a capture agent that specifically binds to a c-erbB-2 peptide.

As used herein, an immunoassay is an assay that utilizes an antibody (*e.g.* a C6 antibody) to specifically bind an analyte (*e.g.*, c-erbB-2). The immunoassay is characterized by the use of specific binding to a C6 antibody as opposed to other physical or chemical properties to isolate, target, and quantify the c-erbB-2 analyte.

The c-erbB-2 marker may be detected and quantified using any of a number of well recognized immunological binding assays. (See for example, U.S. Patents 4,366,241; 4,376,110; 4,517,288; and 4,837,168, which are hereby incorporated by reference.) For a review of the general immunoassays, see also *Methods in Cell Biology Volume 37: Antibodies in Cell Biology*, Asai, ed. Academic Press, Inc. New York (1993); *Basic and Clinical Immunology* 7th Edition, Stites & Terr, eds. (1991)).

The immunoassays of the present invention are performed in any of several configurations, *e.g.*, those reviewed in Maggio (ed.) (1980) *Enzyme Immunoassay* CRC Press, Boca Raton, Florida; Tijan (1985) "Practice and Theory of Enzyme Immunoassays," *Laboratory Techniques in Biochemistry and Molecular Biology*, Elsevier Science Publishers B.V., Amsterdam; Harlow and Lane, *supra*; Chan (ed.) (1987) *Immunoassay: A Practical Guide* Academic Press, Orlando, FL; Price and Newman (eds.) (1991) *Principles and Practice of Immunoassays* Stockton Press, NY; and Ngo (ed.) (1988) *Non isotopic Immunoassays* Plenum Press, NY.

Immunoassays often utilize a labeling agent to specifically bind to and label the binding complex formed by the capture agent and the analyte (*i.e.*, a C6 antibody-erbB-2 complex). The labeling agent may itself be one of the moieties comprising the antibody/analyte complex. Thus, the labeling agent may be a labeled c-erbB-2 peptide or a labeled C6 antibody. Alternatively, the labeling agent is optionally a third moiety, such as another antibody, that specifically binds to the C6 antibody, the c-erbB-2 peptide, the anti-c-erbB-2 antibody/c-erbB-2 peptide complex, or to a modified capture group (*e.g.*, biotin) which is covalently linked to c-erbB-2 or the C6 antibody.

In one embodiment, the labeling agent is an antibody that specifically binds to the C6 antibody. Such agents are well known to those of skill in the art, and

most typically comprise labeled antibodies that specifically bind antibodies of the particular animal species from which the C6 antibody is derived (*e.g.*, an anti-species antibody). Thus, for example, where the capture agent is a human derived C6 antibody, the label agent may be a mouse anti-human IgG, *i.e.*, an antibody specific to the constant region of the human antibody.

Other proteins capable of specifically binding immunoglobulin constant regions, such as streptococcal protein A or protein G are also used as the labeling agent. These proteins are normal constituents of the cell walls of streptococcal bacteria. They exhibit a strong non immunogenic reactivity with immunoglobulin constant regions from a variety of species. See, generally Kronval, *et al.*, (1973) *J. Immunol.*, 111:1401-1406, and Akerstrom, *et al.*, (1985) *J. Immunol.*, 135:2589-2542.

Throughout the assays, incubation and/or washing steps may be required after each combination of reagents. Incubation steps can vary from about 5 seconds to several hours, preferably from about 5 minutes to about 24 hours. However, the incubation time will depend upon the assay format, analyte, volume of solution, concentrations, and the like. Usually, the assays are carried out at ambient temperature, although they can be conducted over a range of temperatures, such as 5°C to 45°C.

#### (a) Non competitive assay formats.

Immunoassays for detecting c-erb-2 are typically either competitive or noncompetitive. Noncompetitive immunoassays are assays in which the amount of captured analyte (in this case, c-erb-2) is directly measured. In one preferred "sandwich" assay, for example, the capture agent (*e.g.*, C6 antibody) is bound directly or indirectly to a solid substrate where it is immobilized. These immobilized C6 antibodies capture c-erb-2 present in a test sample (*e.g.*, a biological sample derived from breast tumor tissue). The c-erb-2 thus immobilized is then bound by a labeling agent, such as a second c-erb-2 antibody bearing a label. Alternatively, the second antibody may lack a label, but it may, in turn, be bound by a labeled third antibody specific to antibodies of the species from which the second antibody is derived. Free labeled antibody is washed away and the remaining bound labeled antibody is detected (*e.g.*, using a gamma detector where the label is radioactive). One of skill will appreciate that the analyte and capture agent is optionally reversed in the above assay,

*e.g.*, when the presence, quantity or avidity of a C6 antibody in a sample is to be measured by its binding to an immobilized c-erbB-2 peptide.

**(b) Competitive assay formats.**

5                    In competitive assays, the amount of analyte (*e.g.*, c-erbB-2) present in the sample is measured indirectly by measuring the amount of an added (exogenous) analyte displaced (or competed away) from a capture agent (*e.g.*, C6 antibody) by the analyte present in the sample. In one competitive assay, a known amount of c-erbB-2 is added to a test sample with an unquantified amount of c-erbB-2, and the sample is contacted with  
10 a capture agent, *e.g.*, a C6 antibody that specifically binds c-erbB-2. The amount of added c-erbB-2 which binds to the C6 antibody is inversely proportional to the concentration of c-erbB-2 present in the test sample.

                    The C6 antibody can be immobilized on a solid substrate. The amount of erbB-2 bound to the C6 antibody is determined either by measuring the amount of erbB-2  
15 present in an erbB-2-C6 antibody complex, or alternatively by measuring the amount of remaining uncomplexed erbB-2. Similarly, in certain embodiments where the amount of erbB-2 in a sample is known, and the amount or avidity of a C6 antibody in a sample is to be determined, erbB-2 becomes the capture agent (*e.g.*, is fixed to a solid substrate) and the C-6 antibody becomes the analyte.

20

**(c) Reduction of Non Specific Binding.**

                    One of skill will appreciate that it is often desirable to reduce non specific binding in immunoassays and during analyte purification. Where the assay involves c-erbB-2, C6 antibody, or other capture agent immobilized on a solid substrate, it is  
25 desirable to minimize the amount of non specific binding to the substrate. Means of reducing such non specific binding are well known to those of skill in the art. Typically, this involves coating the substrate with a proteinaceous composition. In particular, protein compositions such as bovine serum albumin (BSA), nonfat powdered milk, and gelatin are widely used.

30

**(d) Substrates.**

As mentioned above, depending upon the assay, various components, including the erbB-2, C6 or antibodies to erbB-2 or C6, are optionally bound to a solid surface. Many methods for immobilizing biomolecules to a variety of solid surfaces are known in the art. For instance, the solid surface may be a membrane (*e.g.*, nitrocellulose), a microtiter dish (*e.g.*, PVC, polypropylene, or polystyrene), a test tube (glass or plastic), a dipstick (*e.g.* glass, PVC, polypropylene, polystyrene, latex, and the like), a microcentrifuge tube, or a glass, silica, plastic, metallic or polymer bead. The desired component may be covalently bound, or noncovalently attached through nonspecific bonding.

A wide variety of organic and inorganic polymers, both natural and synthetic may be employed as the material for the solid surface. Illustrative polymers include polyethylene, polypropylene, poly(4-methylbutene), polystyrene, polymethacrylate, poly(ethylene terephthalate), rayon, nylon, poly(vinyl butyrate), polyvinylidene difluoride (PVDF), silicones, polyformaldehyde, cellulose, cellulose acetate, nitrocellulose, and the like. Other materials which may be employed, include paper, glassess, ceramics, metals, metalloids, semiconductive materials, cements or the like. In addition, substances that form gels, such as proteins (*e.g.*, gelatins), lipopolysaccharides, silicates, agarose and polyacrylamides can be used. Polymers which form several aqueous phases, such as dextrans, polyalkylene glycols or surfactants, such as phospholipids, long chain (12-24 carbon atoms) alkyl ammonium salts and the like are also suitable. Where the solid surface is porous, various pore sizes may be employed depending upon the nature of the system.

In preparing the surface, a plurality of different materials may be employed, *e.g.*, as laminates, to obtain various properties. For example, protein coatings, such as gelatin can be used to avoid non specific binding, simplify covalent conjugation, enhance signal detection or the like.

If covalent bonding between a compound and the surface is desired, the surface will usually be polyfunctional or be capable of being polyfunctionalized. Functional groups which may be present on the surface and used for linking can include carboxylic acids, aldehydes, amino groups, cyano groups, ethylenic groups, hydroxyl groups, mercapto groups and the like. The manner of linking a wide variety of compounds to various surfaces is well known and is amply illustrated in the literature.

See, for example, *Immobilized Enzymes*, Ichiro Chibata, Halsted Press, New York, 1978, and Cuatrecasas, *J. Biol. Chem.* 245 3059 (1970).

In addition to covalent bonding, various methods for noncovalently binding an assay component can be used. Noncovalent binding is typically nonspecific  
5 absorption of a compound to the surface. Typically, the surface is blocked with a second compound to prevent nonspecific binding of labeled assay components. Alternatively, the surface is designed such that it nonspecifically binds one component but does not significantly bind another. For example, a surface bearing a lectin such as Concanavalin A will bind a carbohydrate containing compound but not a labeled protein that lacks  
10 glycosylation. Various solid surfaces for use in noncovalent attachment of assay components are reviewed in U.S. Patent Nos. 4,447,576 and 4,254,082.

#### ii) Other Assay Formats

C-erbB-2 polypeptides or C6 antibodies and can also be detected and  
15 quantified by any of a number of other means well known to those of skill in the art. These include analytic biochemical methods such as spectrophotometry, radiography, electrophoresis, capillary electrophoresis, high performance liquid chromatography (HPLC), thin layer chromatography (TLC), hyperdiffusion chromatography, and the like, and various immunological methods such as fluid or gel precipitin reactions,  
20 immunodiffusion (single or double), immunoelectrophoresis, radioimmunoassays (RIAs), enzyme-linked immunosorbent assays (ELISAs), immunofluorescent assays, and the like.

Western blot analysis and related methods can also be used to detect and  
25 quantify the presence of erbB-2 peptides and C6 antibodies in a sample. The technique generally comprises separating sample products by gel electrophoresis on the basis of molecular weight, transferring the separated products to a suitable solid support, (such as a nitrocellulose filter, a nylon filter, or derivatized nylon filter), and incubating the sample with the antibodies that specifically bind either the erbB-2 peptide or the anti-erbB-2 antibody. The antibodies specifically bind to the biological agent of interest on  
30 the solid support. These antibodies are directly labeled or alternatively are subsequently detected using labeled antibodies (*e.g.*, labeled sheep anti-human antibodies where the antibody to a marker gene is a human antibody) which specifically bind to the antibody which binds either anti-erbB-2 or erbB-2 as appropriate.

Other assay formats include liposome immunoassays (LIAs), which use liposomes designed to bind specific molecules (*e.g.*, antibodies) and release encapsulated reagents or markers. The released chemicals are then detected according to standard techniques (see, Monroe *et al.*, (1986) *Amer. Clin. Prod. Rev.* 5:34-41).

5

### iii) Labeling of C6 antibodies.

The labeling agent can be, *e.g.*, a monoclonal antibody, a polyclonal antibody, a protein or complex such as those described herein, or a polymer such as an affinity matrix, carbohydrate or lipid. Detection proceeds by any known method, including immunoblotting, western analysis, gel-mobility shift assays, tracking of radioactive or bioluminescent markers, nuclear magnetic resonance, electron paramagnetic resonance, stopped-flow spectroscopy, column chromatography, capillary electrophoresis, or other methods which track a molecule based upon an alteration in size and/or charge. The particular label or detectable group used in the assay is not a critical aspect of the invention. The detectable group can be any material having a detectable physical or chemical property. Such detectable labels have been well-developed in the field of immunoassays and, in general, any label useful in such methods can be applied to the present invention. Thus, a label is any composition detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical or chemical means. Useful labels in the present invention include magnetic beads (*e.g.* Dynabeads™), fluorescent dyes (*e.g.*, fluorescein isothiocyanate, Texas red, rhodamine, and the like), radiolabels (*e.g.*, <sup>3</sup>H, <sup>125</sup>I, <sup>35</sup>S, <sup>14</sup>C, or <sup>32</sup>P), enzymes (*e.g.*, LacZ, CAT, horse radish peroxidase, alkaline phosphatase and others, commonly used as detectable enzymes, either as marker gene products or in an ELISA), and colorimetric labels such as colloidal gold or colored glass or plastic (*e.g.* polystyrene, polypropylene, latex, *etc.*) beads.

The label may be coupled directly or indirectly to the desired component of the assay according to methods well known in the art. As indicated above, a wide variety of labels may be used, with the choice of label depending on the sensitivity required, ease of conjugation of the compound, stability requirements, available instrumentation, and disposal provisions.

Non radioactive labels are often attached by indirect means. Generally, a ligand molecule (*e.g.*, biotin) is covalently bound to the molecule. The ligand then binds to an anti-ligand (*e.g.*, streptavidin) molecule which is either inherently detectable or

covalently bound to a signal system, such as a detectable enzyme, a fluorescent compound, or a chemiluminescent compound. A number of ligands and anti-ligands can be used. Where a ligand has a natural anti-ligand, for example, biotin, thyroxine, and cortisol, it can be used in conjunction with the labeled, naturally occurring anti-ligands.  
5 Alternatively, any haptenic or antigenic compound can be used in combination with an antibody.

The molecules can also be conjugated directly to signal generating compounds, *e.g.*, by conjugation with an enzyme or fluorophore. Enzymes of interest as labels will primarily be hydrolases, particularly phosphatases, esterases and glycosidases,  
10 or oxidoreductases, particularly peroxidases. Fluorescent compounds include fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, *etc.* Chemiluminescent compounds include luciferin, and 2,3-dihydrophthalazinediones, *e.g.*, luminol. For a review of various labelling or signal producing systems which may be used, see, U.S. Patent No. 4,391,904, which is incorporated herein by reference.

15 Means of detecting labels are well known to those of skill in the art. Thus, for example, where the label is a radioactive label, means for detection include a scintillation counter or photographic film as in autoradiography. Where the label is a fluorescent label, it may be detected by exciting the fluorochrome with the appropriate wavelength of light and detecting the resulting fluorescence, *e.g.*, by microscopy, visual  
20 inspection, via photographic film, by the use of electronic detectors such as charge coupled devices (CCDs) or photomultipliers and the like. Similarly, enzymatic labels may be detected by providing appropriate substrates for the enzyme and detecting the resulting reaction product. Finally, simple colorimetric labels may be detected simply by observing the color associated with the label. Thus, in various dipstick assays,  
25 conjugated gold often appears pink, while various conjugated beads appear the color of the bead.

Some assay formats do not require the use of labeled components. For instance, agglutination assays can be used to detect the presence of C6 antibodies and C6 antibody-erbB-2 peptides. In this case, antigen-coated particles are agglutinated by  
30 samples comprising the target antibodies. In this format, none of the components need be labeled and the presence of the target antibody is detected by simple visual inspection.

## V. Pharmaceutical Compositions.

The chimeric molecules of this invention are useful for parenteral, topical, oral, or local administration, such as by aerosol or transdermally, for prophylactic and/or therapeutic treatment. The pharmaceutical compositions can be administered in a variety of unit dosage forms depending upon the method of administration. For example, unit dosage forms suitable for oral administration include powder, tablets, pills, capsules and lozenges. It is recognized that the fusion proteins and pharmaceutical compositions of this invention, when administered orally, must be protected from digestion. This is typically accomplished either by complexing the protein with a composition to render it resistant to acidic and enzymatic hydrolysis or by packaging the protein in an appropriately resistant carrier such as a liposome. Means of protecting proteins from digestion are well known in the art.

The pharmaceutical compositions of this invention are particularly useful for parenteral administration, such as intravenous administration or administration into a body cavity or lumen of an organ. The compositions for administration will commonly comprise a solution of the chimeric molecule dissolved in a pharmaceutically acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers can be used, *e.g.*, buffered saline and the like. These solutions are sterile and generally free of undesirable matter. These compositions may be sterilized by conventional, well known sterilization techniques. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents, toxicity adjusting agents and the like, for example, sodium acetate, sodium chloride, potassium chloride, calcium chloride, sodium lactate and the like. The concentration of chimeric molecule in these formulations can vary widely, and will be selected primarily based on fluid volumes, viscosities, body weight and the like in accordance with the particular mode of administration selected and the patient's needs.

Thus, a typical pharmaceutical composition for intravenous administration would be about 0.1 to 10 mg per patient per day. Dosages from 0.1 up to about 100 mg per patient per day may be used, particularly when the drug is administered to a secluded site and not into the blood stream, such as into a body cavity or into a lumen of an organ. Methods for preparing parenterally administrable compositions will be known or apparent to those skilled in the art and are described in more detail in such publications

as *Remington's Pharmaceutical Science*, 15th ed., Mack Publishing Company, Easton, Pennsylvania (1980).

5 The compositions containing the present fusion proteins or a cocktail thereof (*i.e.*, with other proteins) can be administered for therapeutic treatments. In therapeutic applications, compositions are administered to a patient suffering from a disease, typically a c-erbB-2 positive carcinoma, in an amount sufficient to cure or at least partially arrest the disease and its complications. An amount adequate to accomplish this is defined as a "therapeutically effective dose." Amounts effective for this use will depend upon the severity of the disease and the general state of the patient's health.

10 Single or multiple administrations of the compositions may be administered depending on the dosage and frequency as required and tolerated by the patient. In any event, the composition should provide a sufficient quantity of the proteins of this invention to effectively treat the patient.

15 Among various uses of the cytotoxic fusion proteins of the present invention are included a variety of disease conditions caused by specific human cells that may be eliminated by the toxic action of the protein. One application is the treatment of cancer, such as by the use of a C6 antibody attached to a cytotoxin.

20 Another approach involves using a ligand that binds a cell surface marker (receptor) so the chimeric associates cells bearing the ligand substrate are associated with the c-erbB-2 overexpressing tumor cell. The ligand portion of the molecule is chosen according to the intended use. Proteins on the membranes of T cells that may serve as targets for the ligand includes Fc $\gamma$ I, Fc $\gamma$ II and Fc $\gamma$ III, CD2 (T11), CD3, CD4 and CD8. Proteins found predominantly on B cells that might serve as targets include CD10 (CALLA antigen), CD19 and CD20. CD45 is a possible target that occurs broadly on lymphoid cells. These and other possible target lymphocyte target molecules for the chimeric molecules bearing a ligand effector are described in *Leukocyte Typing III*, A.J. McMichael, ed., Oxford University Press (1987). Those skilled in the art will realize ligand effectors may be chosen that bind to receptors expressed on still other types of cells as described above, for example, membrane glycoproteins or ligand or hormone receptors such as epidermal growth factor receptor and the like.

## VI. Kits For Diagnosis or Treatment.

In another embodiment, this invention provides for kits for the treatment of tumors or for the detection of cells overexpressing c-erbB-2. Kits will typically comprise a chimeric molecule of the present invention (*e.g.* C6 antibody-label, C6 antibody-cytotoxin, C6 antibody-ligand, *etc.*). In addition the kits will typically include instructional materials disclosing means of use of chimeric molecule (*e.g.* as a cytotoxin, for detection of tumor cells, to augment an immune response, *etc.*). The kits may also include additional components to facilitate the particular application for which the kit is designed. Thus, for example, where a kit contains a chimeric molecule in which the effector molecule is a detectable label, the kit may additionally contain means of detecting the label (*e.g.* enzyme substrates for enzymatic labels, filter sets to detect fluorescent labels, appropriate secondary labels such as a sheep anti-human antibodies, or the like). The kits may additionally include buffers and other reagents routinely used for the practice of a particular method. Such kits and appropriate contents are well known to those of skill in the art.

### EXAMPLES

The following examples are provided by way of illustration only and not by way of limitation. Those of skill will readily recognize a variety of noncritical parameters which could be changed or modified to yield essentially similar results.

#### Example 1

##### Isolation and Characterization of Human Single-chain Fvs Binding C-erbb-2

###### Materials and Methods:

###### *Preparation of c-erbB-2 ECD*

The antigen c-erbB-2 ECD with a Ser-Gly-His<sub>6</sub> C-terminal fusion was expressed from Chinese Hamster Ovary cells and purified by immobilized metal affinity chromatography (IMAC).

###### *Phage preparation*

Phage were prepared from a phagemid library ( $3 \times 10^7$  members) expressing sFv as pIII fusions on the phage surface (Marks *et al.* (1991) *J. Mol. Biol.* 222:581-597). The library was created from a repertoire of sFv genes consisting of

human heavy and light chain variable region ( $V_H$  and  $V_L$ ) genes isolated from the peripheral blood lymphocytes of unimmunized human volunteers. To rescue phagemid particles from the library, 50 ml of 2 x TY media containing 100  $\mu\text{g/ml}$  ampicillin and 1% glucose were inoculated with  $10^8$  bacteria taken from the frozen library glycerol stock. The culture was grown at 37°C with shaking to an  $A_{600}$  nm of 0.8,  $7.0 \times 10^{11}$  colony forming units of VCS-MI3 (Stratgene) added, and incubation continued at 37°C for 1 h without shaking followed by 1 h with shaking. The cells were pelleted by centrifugation at 4500g for 10 min, resuspended in 200 ml of 2 x TY media containing 100  $\mu\text{g/ml}$  ampicillin and 2.5  $\mu\text{g/ml}$  kanamycin and grown overnight at 37°C. Phage particles were purified and concentrated by 2 polyethylene glycol precipitations and resuspended in PBS (25 mM  $\text{NaH}_2\text{PO}_4$ , 125 mM NaCl, pH 7.0) to approximately  $10^{13}$  transducing units/ml ampicillin resistant clones.

#### *Selection of binding phage antibodies*

Phage expressing sFv which bound c-erbB-2 were selected by panning the phage library on immobilized c-erbB-2 ECD (Marks *et al.* (1991) *supra.*). Briefly, immunotubes (Nunc, Maxisorb) were coated with 2 ml (100  $\mu\text{g/ml}$ ) c-erbB-2 ECD in PBS overnight at 20°C and blocked with 2% milk powder in PBS for 2 h at 37°C. 1 ml of the phage solution (approximately  $10^{13}$  phage) was added to the tubes and incubated at 20°C with tumbling on an over and under turntable for 2 h. Nonbinding phage were eliminated by sequential washing (15 times with PBS containing 0.05% Tween followed by 15 times with PBS). Binding phage were then eluted from the immunotubes by adding 1 ml of 100 mM triethylamine, incubating for 10 min at 20°C, transferring the solution to a new tube, and neutralizing with 0.5 ml 1M Tris HCl, PH 7.4. Half of the eluted phage solution was used to infect 10 ml of *E.coli* TG1 (Gibson, T.J. (1984) *Studies on the Epstein-Barr virus genome*, Cambridge University Ph.D. thesis; Carter *et al.* (1985) *Nucleic Acids Res.*, 13: 4431-4443) grown to an  $A_{600}$  nm of 0.8-0.9. After incubation for 30 min at 37°C, bacteria were plated on TYE plates containing 100  $\mu\text{g/ml}$  ampicillin and 1% glucose and grown overnight at 37°C. Phage were rescued and concentrated as described above and used for the next selection round. The selection process was repeated for a total of 5 rounds.

*Screening for binders*

After each round of selection, 10 ml of *E.coli* HB2151 (Carter *et al.* (1985) *Nucleic Acids Res.*, 13: 4431-43) ( $A_{600}$  run  $\sim$  0.8) were infected with 100  $\mu$ l of the phage eluate in order to prepare soluble sFv. In this strain, the amber codon  
 5 between the sFv gene and gene III is read as a stop codon and native soluble sFv secreted into the periplasm and media (Hoogenboom *et al.* (1991) *Nucleic Acids Res.* 19: 4133-4137). Single ampicillin resistant colonies were used to inoculate microtitre plate wells containing 150  $\mu$ l of 2 x TY containing 100  $\mu$ g/ml ampicillin and 0.1% glucose. The bacteria were grown to an  $A_{600}$  nm  $\sim$  1.0, and sFv expression induced by the  
 10 addition of IPTG to a final concentration of 1 mM (De Bellis *et al.*, (1990) *Nucleic Acids Res.*, 18:1311). Bacteria were grown overnight at 30°C, the cells removed by centrifugation, and the supernatant containing sFv used directly.

To screen for binding, 96-well microtiter plates (Falcon 3912) were coated overnight at 4°C with 10  $\mu$ g/ml c-erbB-2 ECD in PBS, blocked for 2 h at 37°C with 2%  
 15 milk powder in PBS, and incubated for 1.5 hours at 20°C with 50  $\mu$ l of the *E.coli* supernatant containing sFv. Binding of soluble sFv to antigen was detected with a mouse monoclonal antibody (9E1O) which recognizes the C-terminal myc peptide tag (Munro, S. *et al.*, (1986) *Cell*, 46:291-300) and peroxidase conjugated anti-mouse Fc antibody (Sigma) using ABTS as substrate (Ward *et al.* (1989) *Nature*, 341: 544-546). The  
 20 reaction was stopped after 30 min with NaF (3.2 mg/ml) and the  $A_{405}$  nm measured. Unique clones were identified by PCR fingerprinting (Marks, J. D. *et al.*, (1991) *J. Mol. Biol.*, 222:581-597) and DNA sequencing. The specificity of each unique sFv was determined by ELISA performed as described above with wells coated with 10  $\mu$ g/ml of  
 25 bovine serum albumin, hen egg white lysozyme, bovine glutamyltranspeptidase, c-erbB-2 ECD, VCS M13 ( $3.5 \times 10^{12}$ /ml) and casein (0.5%). For ELISA with biotinylated c-erbB-2 ECD, microtiter plates (Immunolon 4, Dynatech) were coated with 50  $\mu$ l immunopure avidin (Pierce; 10  $\mu$ g/ml in PBS) overnight at 4°C, blocked with 1% bovine serum albumin in PBS for 1 h at 37°C and incubated with 50  $\mu$ l biotinylated c-erbB-2 extracellular domain (5  $\mu$ g/ml) for 30 min at 20°C. To prepare biotinylated antigen, 0.2  
 30 ml c-erbB-2 ECD (1 mg/ml in PBS) was incubated with 0.5 mM NHS-LC-biotin (Pierce) overnight at 4°C and then purified on a presto desalting column (Pierce).

*Subcloning, expression and purification.*

To facilitate purification, the C6.5 sFv gene was subcloned into the expression vector pUC119Sfi/NotHismyc (Griffiths, *et al.* (1994) *EMBO J.*, 13: 3245-3260) which results in the addition of a hexa-histidine tag at the C-terminal end of the sFv. Briefly, pHEN-1 vector DNA containing the C6.5 sFv DNA was prepared by alkaline lysis milliprep, digested with NcoI and NotI, and the sFv DNA purified on a 1.5% agarose gel. C6.5 sFv DNA was ligated into pUC119Sfi/NotHismyc digested with NcoI and NotI and the ligation mixture used to transform electrocompetent *E. coli* HB2151. For expression, 200 ml of 2 x TY media containing 100 µg/ml ampicillin and 0.1% glucose was inoculated with *E. coli* HB2151 harboring the C6.5 gene in pUC119Sfi/NotHismyc. The culture was grown at 37°C to an A<sub>600</sub> nm of 0.8, soluble sFv expression induced by the addition of IPTG to a final concentration of 1 mM, and the culture grown at 30°C in a shaker flask overnight. Single-chain Fv was harvested from the periplasm using the following protocol. Cells were harvested by centrifugation at 4000g for 15 min, resuspended in 10 ml of ice cold 30 mM Tris-HCl pH 8.0, 1 mM EDTA, 20% sucrose, and incubated on ice for 20 min. The bacteria were pelleted by centrifugation at 6000g for 15 min. and the "periplasmic fraction" cleared by centrifugation at 30,000g for 20 min. The supernatant was dialyzed overnight at 4°C against 8 L of IMAC loading buffer (30 mM sodium phosphate pH 7.5, 500 mM NaCl, 20 mM imidazole) and then filtered through a 0.2 micron filter.

The sFv was purified by IMAC. All steps were performed at 4°C on a Perceptive Biosystems BIOCAD Sprint. A column containing 2 ml of Ni-NTA resin (Qiagen) was washed with 20 ml IMAC column wash buffer (50 mM sodium phosphate pH 7.5, 500 mM NaCl, 2.50 mM imidazole) and 20 ml of IMAC loading buffer. The periplasmic preparation was loaded onto the column by pump and the column washed sequentially with 50 ml IMAC loading buffer and 50 ml IMAC washing buffer (50 mM sodium phosphate pH 7.5, 500 mM NaCl, 23 mM imidazole). Protein was eluted with 2.5 ml IMAC elution buffer (50 mM sodium phosphate pH 7.5, 300 mM NaCl, 100 mM imidazole) and 4 ml fractions collected. Protein was detected by absorbance at 280 nm and sFv typically eluted between fractions 6 and 8. To remove dimeric and aggregated sFv, samples were concentrated to a volume < 1 ml in a Centricon 10 (Amicon) and fractionated on a Superdex 75 column using a running buffer of HBS (10 mM Hepes, 150 mM NaCl, pH 7.4). The purity of the final preparation was evaluated by assaying an

aliquot by SDS-PGE. Protein bands were detected by Coomassie staining. The concentration was determined spectrophotometrically, assuming an  $A_{280}$  run of 1.0 corresponds to an sFv concentration of 0.7 mg/ml.

#### 5            *Affinity and kinetic measurements*

The  $K_d$  of C6.5 and 741F8 sFv were determined using surface plasmon resonance in a BIAcore (Pharmacia) and by Scatchard analysis. In a BIAcore flow cell, 1400 resonance units (RU) of c-erbB-2 ECD (25  $\mu$ g/ml in 10 mM sodium acetate, pH 4.5) was coupled to a CM5 sensor chip (Johnsson, B. et al., (1991) *Anal. Biochem.*, 198:268-277). Association and dissociation of C6.5 and 741F8 sFv (100 nM - 600 nM) were measured under continuous flow of 5  $\mu$ l/min. Rate constant  $k_{on}$  was determined from a plot of  $(1/\ln(dR/dt))/t$  vs concentration (Karlsson *et al.* (1991) *J. Immunol. Meth.*, 145: 229-240). Rate constant  $k_{off}$  was determined from the dissociation part of the sensorgram at the highest concentration of sFv analyzed (Johnsson *et al.* (1991) *Anal. Biochem.*, 198: 268-277). The  $K_d$  of C6.5 was also determined by Scatchard analysis (Scatchard (1949) *Annal. N.Y. Acad. Sci.*, 51: 660). All assays were performed in triplicate. Briefly, 50  $\mu$ g of radioiodinated sFv was added to  $5 \times 10^6$  SK-OV-3 cells in the presence of increasing concentrations of unlabeled sFv from the same preparation. After a 30 minute incubation at 20°C, the samples were washed with PBS at 40°C and centrifuged at 500g. The amount of labeled sFv bound to the cells was determined by counting the pellets in a gamma counter and the  $K_a$  and  $K_d$  were calculated using the EBDA program (V 2.0, G.A. McPherson, 1983).

#### *Radiolabeling*

25            The C6.5 sFv was labeled with radioiodine using the CT method (DeNardo *et al.* (1986) *Nud. Med. Biol.*, 13: 303-310). Briefly, 1.0 mg of protein was combined with  $^{125}$ I (14-17 mCi/mg) (Amersham, Arlington Heights, IL), or  $^{131}$ I (9.25 mCi/mg) (DuPont NEN, Wilmington, DE) at an iodine to protein ratio of 1:10. 10  $\mu$ g of CT (Sigma, St. Louis, MO) was added per 100  $\mu$ g of protein and the resulting mixture was incubated for three minutes at room temperature. The reaction was quenched by the addition of 10  $\mu$ g of sodium metabisulfite (Sigma) per 100  $\mu$ g of protein. Unincorporated radioiodine was separated from the labeled protein by gel filtration using the G-50-80 centrifuged-column method (Adams *et al.* (1993) *Cancer*

*Res.* 53: 4026-4034). The final specific activity of the CT labelling was 1.4 mCi/mg for the  $^{131}\text{I}$ -C6.5 sFv and typically about 1.0 mCi/mg for the  $^{125}\text{I}$ -C6.5 sFv.

### *Quality Control*

5           The quality of the radiopharmaceuticals was evaluated by HPLC, SDS-PAGE, and a live cell binding assay as previously described (Adams *et al.* (1993) *Cancer Res.* 53: 4026-4034). The HPLC elution profiles from a Spherogel TSK-3000 molecular sieving column consistently demonstrated that greater than 99% of the radioactivity was associated with the protein peak. Greater than 98% of the nonreduced  
10  $^{125}\text{I}$ -C6.5 sFv preparations migrated on SDS-PAGE as approximately 26 K<sub>d</sub> proteins while the remaining activity migrated as a dimer. The immunoreactivity of the radiopharmaceuticals was determined in a live cell binding assay utilizing c-erbB-2 overexpressing SK-OV-3 cells (#HTB 77; American Type Culture Collection, Rockville, MD) and c-erbB-2 negative CEM cells (#119; American Type Culture Collection)  
15 (Adams, G.P. et al., (1993) *Cancer Res.* 53:4026-4034). Live cell binding assays revealed 49% of the activity associated with the positive cell pelleted less than 3% bound to the negative control cells; these results were lower than those typically seen with 741F8 sFv (60-80% bound) (Adams *et al.*, (1993) *supra.*).

### 20           *Cell Surface Dissociation Studies*

Cell surface retention of biotinylated forms of the sFv molecules were measured by incubating 2  $\mu\text{g}$  of either sFv with  $2 \times 10^6$  SK-BR-3 cells (#HTB 30; American Type Culture Collection) in triplicate in 20 ml of FACS buffer, with 0.01% azide for 15 min at 4°C. The cells were washed twice with FACS buffer (4°C) and  
25 resuspended in 2 ml of FACS buffer. 0.5 ml of the cell suspension were removed and placed in three separate tubes for incubations under differing conditions; 0 min at 4°C, 15 min at 37°C, and 30 min at 37°C. After the incubations, the cells were centrifuged at 300g, the supernatants were removed, the cell pellets were washed 2x (4°C) and the degree of retention of sFv on the cell surface at 37°C (for 15 or 30 min) was compared  
30 to retention at 0 min at 4°C.

*Biodistribution and Radioimmunoimaging Studies*

Four to six week old C.B17/Icr-*scid* mice were obtained from the Fox Chase Cancer Center Laboratory Animal Facility.  $2.5 \times 10^6$  SK-OV-3 cells in log phase were implanted s.c. on the abdomens of the mice. After about 7 weeks the tumors had achieved sizes of 100-200 mg and Lugol's solution was placed in the drinking water to block thyroid accumulation of radioiodine. Three days later, biodistribution studies were initiated.  $^{125}\text{I}$ -C6.5 sFv was diluted in PBS to a concentration of 0.2 mg/ml and each mouse was given 100  $\mu\text{L}$ , containing 20  $\mu\text{g}$  of radiopharmaceutical, by tail vein injection. Total injected doses were determined by counting each animal on a Series 30 multichannel analyzer/probe system (probe model #2007, Canaberra, Meridian, CT). Blood samples and whole body counts of the mice were obtained at regular intervals. Groups of 8 mice were sacrificed at 24 h after injection and the tumors and organs removed, weighed and counted in a gamma counter to determine the %ID/g (Adams *et al.* (1993) *supra.*; Adams *et al.* (1992) *Antibody Immunocnj. and Radiopharm.*, 5: 81-95). The mean and standard error of the mean (SEM) for each group of data were calculated, and T:O ratios determined. Significance levels were determined using Students t-test.

For the radioimmunoimaging studies, tumor-bearing *scid* mice were injected with 100  $\mu\text{g}$  (100  $\mu\text{l}$ ) of  $^{131}\text{I}$ -C6.5. At 24 hours after injection, the mice were euthanized by asphyxiation with  $\text{CO}_2$  and images were acquired on a Prism 2000XP gamma camera (Picker, Highland Heights, OH 44142). Preset acquisitions of 100k counts were used.

Table 6  
 Deduced amino acid sequence of C4.1 and C4.5 heavy and light chains. Sequences are aligned to the most homologous human germline gene. Dashes indicate sequence identity. GL = germline gene sequence. DP58 and DP73 [22], KGLV122 and DP15 [24]

Heavy chains	Framework 1	CDR1	Framework 2	CDR2	Framework 3	CDR3	Framework 4
C4.1	QMLVDSGGELMGGSLM	SYEM	WYDQAKGGLNWS	YISSGSPHYAGSNG	KPTISDAWAGSLYORSLAEDATVATYCAR	DLEGTSTGYAGLDY	WQQTLLVTVSS
DP58	QMLVDSGGELMGGSLM	SYEM	WYDQAKGGLNWS	YISSGSPHYAGSNG	KPTISDAWAGSLYORSLAEDATVATYCAR	DLEGTSTGYAGLDY	WQQTLLVTVSS
C4.5	QVLLQSGELAKNGELM	ISCMOSGTSPT	SYVLA	WVHQKNGGLNWS	LTYGSDITNYSPTG	QVTISVAKSVSTAYLQVSSSLPISDSAVYFCAR	HWQKSSSGLCAWRPEYTCM
DP73	QVLLQSGELAKNGELM	ISCMOSGTSPT	SYVLA	WVHQKNGGLNWS	LTYGSDITNYSPTG	QVTISVAKSVSTAYLQVSSSLPISDSAVYFCAR	HWQKSSSGLCAWRPEYTCM
Light chains	Framework 1	CDR1	Framework 2	CDR2	Framework 3	CDR3	Framework 4
C4.1	SELVQIQRVSVLQVPRITC	QGSLSASTAS	WYQKPKQAPLVLY	CHQKYS	GIPDQPSGSSSCHIASLPTTCQAEDEADNYC	MSDSSSCEIRVAV	FOGGTAVTVLG
KGLV121	SELVQIQRVSVLQVPRITC	QGSLSASTAS	WYQKPKQAPLVLY	CHQKYS	GIPDQPSGSSSCHIASLPTTCQAEDEADNYC	MSDSSSCEIRVAV	FOGGTAVTVLG
C4.5	QSVLTQVPSVSAAPQVITTC	SGSSMIGRATVS	WYQQLPQVAPLVLY	CHTRVPA	QVFDVPSGSKSGTSAASLAIISCFASIEDEADNYC	AKWDSLSG HW	FOGGTAVTVLG
HWLV172	QSVLTQVPSVSAAPQVITTC	SGSSMIGRATVS	WYQQLPQVAPLVLY	CHTRVPA	QVFDVPSGSKSGTSAASLAIISCFASIEDEADNYC	AKWDSLSG HW	FOGGTAVTVLG
DP15	QSVLTQVPSVSAAPQVITTC	SGSSMIGRATVS	WYQQLPQVAPLVLY	CHTRVPA	QVFDVPSGSKSGTSAASLAIISCFASIEDEADNYC	AKWDSLSG HW	FOGGTAVTVLG

## Results

After four rounds of selection, 9/190 clones analyzed by ELISA expressed sFv which bound c-erbB-2 ECD (ELISA signals greater than 0.4, 6 times higher than background). After five rounds of selection, 33/190 clones expressed c-erbB-2 binding sFv. PCR fingerprinting of the 42 positive clones identified two unique restriction patterns and DNA sequencing of 6 clones from each pattern revealed two unique human sFv sequences, C4.1 and C6.5 (Table 6). The  $V_H$  gene of C6.5 is from the human  $V_{H5}$  gene family, and the  $V_L$  gene from the human  $V_{\lambda}$  family (Table 6). The  $V_L$  gene appears to be derived from two different germline genes (HUMLV122 and DPL 5) suggesting the occurrence of PCR crossover (Table 6). The  $V_H$  gene of C4.1 is from the human  $V_{H3}$  family, and the  $V_L$  gene from the human  $V_{\lambda 3}$  family (Table 6). C4.1 and C6.5 both bound c-erbB-2 specifically, as determined by ELISA against the relevant antigen and a panel of irrelevant antigens. However, when biotinylated c-erbB-2 ECD was bound to avidin coated plates and used in ELISA assays, the signal obtained with C6.5 was 6 times higher than observed when c-erbB-2 ECD was absorbed to polystyrene (1.5 vs 0.25). In contrast, C4.1 was not capable of binding to biotinylated c-erbB-2 ECD captured on avidin microtitre plates. Additionally, biotinylated and iodinated C6.5, but not C4.1, bound SK-BR-3 cells overexpressing c-erbB-2. These results indicate that C6.5 binds the native c-erbB-2 expressed on cells, but C4 binds a denatured epitope that appears when the antigen is adsorbed to polystyrene.

C6.5 was purified in yields of 10 mg/L of *E. coli* grown in shake flasks and gel filtration analysis indicated a single peak of approximately 27  $K_d$ . The  $K_d$  of purified C6.3 was determined using both surface plasmon resonance in a BIAcore and by Scatchard. The  $K_d$  determined by BIAcore ( $1.6 \times 10^{-8}$  M) agreed closely to the value determined by Scatchard ( $2.0 \times 10^{-8}$  M) (Table 7). Kinetic analysis by BIAcore indicated that C6.5 had a rapid on-rate ( $k_{on}$   $4.0 \times 10^5 M^{-1}s^{-1}$ ) and a rapid off-rate ( $k_{off}$   $6.3 \times 10^{-3} s^{-1}$ ) (Table 2). Cell retention assay confirmed that C6.5 dissociated rapidly from the cell surface (Table 2).

After injection of  $^{125}I$ -C6.5 into *scid* mice bearing SK-OV-3 tumors, 1.47% ID/gm of tumor was retained after 24 hours (Table 7). Tumor:normal organ values ranged from 8.9 (tumor:kidney) to 283 (tumor:muscle). These values were higher than values observed for 741F8 sFv, produced from a murine monoclonal antibody ( $K_d = 2.6$

$\times 10^{-8}$  M. The high T:O ratios resulted in the highly specific visualization of the tumor by gamma scintigraphy using  $^{131}\text{I}$ -labelled C6.5.

Table 7. **Characterization of anti-cerbB-2 sFv species.** Characteristics of the murine anti-c-erbB-2 sFv, 741F8, and the human sFv C6.5 are compared. The affinity and dissociation constants were determined by Scatchard plot analysis, unless otherwise stated. Dissociation from c-erbB-2 positive (SK-OV-3) cells was measured in an in vitro live cell assay. The percentage of injected dose per gram (%ID/g) tumor M and tumor to organ ratios were determined in biodistribution studies performed in separate groups of scid mice (n=10-14) bearing SK-OV-3 tumors overexpressing c-erbB-2. SEM are < 35% of the associated values a = significantly unproved ( $p < 0.05$ ) compared to 741F8 sFv.

Parameter	741F8	C6.5
$K_d$ (BIAcore)	$2.6 \times 10^{-8} \text{M}$	$1.6 \times 10^{-8} \text{M}$
$K_d$ (Scatchard)	$5.4 \times 10^{-8} \text{M}$	$2.1 \times 10^{-8} \text{M}$
$k_{on}$ (BIAcore)	$2.4 \times 10^5 \text{M}^{-1} \text{s}^{-1}$	$4.0 \times 10^5 \text{M}^{-1} \text{s}^{-1}$
$k_{off}$ (BIAcore)	$6.4 \times 10^{-3} \text{s}^{-1}$	$6.3 \times 10^{-3} \text{s}^{-1}$
% associated with cell surface at 15 min	32.7%	60.6%
% associated with cell surface at 15 min	8.6%	22.2%
%ID/g Tumor	0.8	1.0
T:Blood	14.7	22.9
T:Kidney	2.8	5.6a
T:Liver	14.2	22.3
T:Spleen	10.3	34.1
T:Intestine	25.0	29.7
T:Lung	9.4	15.8
T:Stomach	8.9	11.1
T:Muscle	78.8	158.7
T:Bone	30.0	102.7

**Example 2****Isolation of High Affinity Monomeric Human Anti-cerb-2 Single Chain Fv Using Affinity Driven Selection****Materials and Methods**5            *Construction of heavy chain shuffled libraries*

To facilitate heavy chain shuffling, libraries were constructed in pHEN-1 (Hoogenboom *et al.* (1991) *Nucleic Acids Res.* 19, 4133-4137) containing human V<sub>H</sub> gene repertoires (FR1 to FR3) and a cloning site at the end of V<sub>H</sub> FR3 for inserting the V<sub>H</sub> CDR3, V<sub>H</sub> FR4, linker DNA and light chain from binding sFv as a BssHII-NotI  
10 fragment. To create the libraries three V<sub>H</sub> gene repertoires enriched for human V<sub>H1</sub>, V<sub>H3</sub>, and V<sub>H5</sub> gene were amplified by PCR using as a template single stranded DNA prepared from a 1.8 x 10<sup>8</sup> member sFv phage antibody library pHEN-1 (Marks *et al.* (1991) *J. Mol. Biol.* 222: 581-597). For PCR, 50 µl reactions were prepared containing  
15 10 ng template, 25 pmol back primer (LMB3), 25 pmol forward primer (PV<sub>H1</sub>FOR1, PV<sub>H3</sub>FOR1, or PV<sub>H5</sub>FOR1), 250 µM-dNTPs, 1 mM MgCl<sub>2</sub>, and 0.5 µl (2 units) Taq DNA polymerase (Promega) in the manufacturer's buffer. Primers PV<sub>H1</sub>For1, PV<sub>H3</sub>For1, and PV<sub>H5</sub>For1 were designed to anneal to the consensus V<sub>H1</sub>, V<sub>H3</sub>, or, V<sub>H5</sub> 3' FR3 sequence respectively (Tomlinson *et al.* (1992) *J. Mol. Biol.* 227, 776-798; see Table 18). The reaction mixture was subjected to 25 cycles of amplification (94°C for  
20 30 sec, 55 °C for 30 sec and 72°C for 30 sec) using a Hybaid OmniGene cycler. The products were gel purified, isolated from the gel using DEAE membranes, eluted from the membranes with high salt buffer, ethanol precipitated, and resuspended in 20 µl of water (Sambrook *et al.* (1990)).

The DNA fragments from the first PCR were used as templates for a  
25 second PCR to introduce a BssHII site at the 3'-end of FR3 followed by a NotI site. The BssHII site corresponds to amino acid residue 93 and 94 (Kabat numbering (Kabat *et al.* (1987) *Sequences of proteins of immunological interest*, 4th ed., US Department of Health and Human Services, Public Health Service, Bethesda, MD; see, Table 5 in this reference) does not change the amino acid sequence (alanine-arginine). PCR was  
30 performed as described above using 200 ng purified first PCR product as template and the back primers PV<sub>H1</sub>For2, PV<sub>H3</sub>For2, and PV<sub>H5</sub>For2. The PCR products were purified by extraction with phenol/chloroform, precipitated with ethanol, resuspended in 50 µl water and 5 µg digested with NotI and NcoI. The digested fragments were gel

purified and each  $V_H$  gene repertoire ligated separately into pHEN-1 (Hoogenboom *et al.* 1991 *supra.*) digested with NotI and NcoI. The ligation mix was purified by extraction with phenol/chloroform, ethanol precipitated, resuspended in 20  $\mu$ l water, and 2.5  $\mu$ l samples electroporated (Dower *et al.* (1988) *Nucleic Acids Res.* 16, 6127-6145) into 50  $\mu$ l *E. coli* TG1 (Gibson *et al.* (1984) Ph.D. Thesis, University of Cambridge). Cells were grown in 1 ml SOC (Sambrook *et al.* 1990) for 3 min and then plated on TYE (Miller (1972) *Experiments in Molecular Genetics* Cold Springs Harbor Lab Press, Cold Springs Harbor, New York) media containing 100  $\mu$ g ampicillin/ml and 1% (w/v) glucose (TYE-AMP-GLU). Colonies were scraped off the plates into 5 ml of 2 x TY broth (Miller (1972), *supra*) containing 100  $\mu$ g ampicillin/ml, 1% glucose (2 x TY-AMP-GLU) and 15 (v/v) glycerol for storage at  $-70^\circ\text{C}$ . The cloning efficiency and diversity of the libraries were determined by PCR screening (Gussow and Clackson (1989) *Nucleic Acids Res.* 17, 4000) as described (Marks *et al.* (1991), *supra*). The resulting phage libraries were termed pHEN-1- $V_H$ 1rep, pHEN-1- $V_H$ 3rep and pHEN-1- $V_H$ 5rep.

Three separate C6.5 heavy chain shuffled phage antibody libraries were made from the pHEN-1- $V_H$ 1rep, pHEN-1- $V_H$ 3rep, and pHEN-1- $V_H$ 5rep phage libraries. The C6.5 light chain gene, linker DNA, and  $V_H$  CDR and FR4 were amplified by PCR from pHEN-1-C6.5 plasmid DNA using the primers PC6VL1Back and fdSEQ1. The PCR reaction mixtures were digested with BssHII and NotI and ligated into pHEN-1- $V_H$ 1rep, pHEN-1- $V_H$ 3rep, and pHEN-1- $V_H$ 5rep digested with NotI and BssHII. Transformation and creation of library stocks was as described above.

#### *Construction of light chain shuffled libraries*

To facilitate light chain shuffling, a library was constructed in PHEN-1 containing human  $V_k$  and  $V_\lambda$  gene repertoires, linker DNA, and cloning sites for inserting a  $V_H$  gene as an NcoI-XhoI fragment. An XhoI can be encoded at the end of FR4 without changing the amino acid sequence of residues 102 and 103 (serine-serine) (Kabat *et al. Sequences of proteins of immunological interest*, 4th ed. U.S. Dept. Health and Human Services, Public Health Services, Bethesda, MD (1987)). To create the library, a  $V_k$  and  $V_\lambda$  gene repertoire was amplified by PCR from a  $1.8 \times 10^8$  member sFv phage antibody library in pHEN-1 (Marks *et al.* (1991), *supra*). PCR was performed as described above using 10 ng template, 25 pmol Back primer (RJH1/2/6Xho, RJH3Xho,

oRJH4/5Xho) and 25 pmol forward primer (fdSEQ1). The back primers were designed to anneal to the first 6 nucleotides of the (G4S) linker and either the J<sub>H</sub>1, 2, 6, J<sub>H</sub>3, or J<sub>H</sub>4,5 segments respectively. The PCR reaction mixture was purified as described above, digested with XhoI and NotI, gel purified and ligated into pHEN-V<sub>λ</sub>3S1 (Hoogenboom and Winter (1992) *J. Mol. Biol.* 227, 381-388) digested with XhoI and NotI.

5

Transformation of *E. coli*, TG1, PCR screening, and creation of library stocks was as described above. The resulting phage library was termed pHEN-1-V<sub>L</sub>rep.

The light chain shuffled phage antibody library was made for pHEN-1-V<sub>L</sub>rep. The C6.5 V<sub>H</sub> gene was amplified by PCR from pHEN-1-C6.5 plasmid DNA using the primers PC6V<sub>H</sub>1For and LMB3. The PCR reaction mixture was purified, digested with XhoI and NcoI, gel purified and ligated into pHEN-1-V<sub>L</sub>rep digested with Xho and NcoI. Transformation of *E. coli* TG1, PCR screening, and creation of library stocks was as described above.

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#### *Construction of sFv containing highest affinity V<sub>H</sub> and V<sub>L</sub> gene obtained by chain shuffling*

Two new sFv were made by combining the V<sub>L</sub> gene of the highest affinity light chain shuffled sFv (C6L1) with the V<sub>H</sub> gene of the highest affinity heavy chain shuffled sFv (C6H1 or C6H2). The C6L1 plasmid was digested with NcoI and XhoI to remove the C6.5 V<sub>H</sub> gene and gel purified. The V<sub>H</sub> gene of C6H1 or C6H2 was amplified by PCR using the primers LMB3 and PC6V<sub>H</sub>1For, digested with NcoI and XhoI and ligated into the previously digested C6L1 vector. Clones were screened for the presence of the correct insert by PCR fingerprinting and confirmed by DNA sequencing.

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25

#### *Preparation of phage*

To rescue phagemid particles from the libraries, 10 ml of 2 TY-AMP-GLU were inoculated with an appropriate volume of bacteria (approximately 50 to 100 μl) from the library stocks to give an A<sub>600</sub> of 0.3 to 0.5 and grown for 30 min, shaking at 37°C. About 1 x 10<sup>12</sup> plaque-forming units of VCS-M13 (Stratagene) particles were added and the mixture incubated at 37°C for 30 min without shaking followed by incubation at 37°C for 30 min with shaking. Cells were spun down, resuspended in 50 ml 2 x TY broth containing 100 μg ampicillin/ml and 50 μg kanamycin/ml (2 x TY-AMP-KAN), and grown overnight, shaking at 25°C. Phage

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particles were purified and concentrated by two PEG-precipitations (Sambrook *et al.*, 1990), resuspended in 5 ml phosphate buffered saline (25 mM NaH<sub>2</sub>PO<sub>4</sub>, 125 mM NaCl, pH 7.0, PBS) and filtered through a 0.45  $\mu$  filter. The phage preparation consistently resulted in a titre of approximately 10<sup>13</sup> transducing units/ml ampicillin-resistant clones.

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#### *Selection of phage antibody libraries*

The light chain shuffled library was selected using immunotubes (Nunc; Maxisorb) coated with 2 ml c-erbB-2 ECD (25  $\mu$ g/ml) in PBS overnight at room temperature (Marks *et al.* (1991) *supra*). The tube was blocked for 1 h at 37°C with 2% skimmed milk powder in PBS (2% MPBS) and the selection, washing, and elution were performed as described (Marks *et al.* (1991), *supra*) using phage at a concentration of 5.0 x 10<sup>12</sup>/ml. One third of the eluted phage was used to infect 1 ml log phase *E. coli* TG1, which were plated on TYE-AMP-GLU plates and described above. The rescue-selection-plating cycle was repeated 3 times, after which clones were analyzed for binding by ELISA.

15

All libraries were also selected using biotinylated c-erbB-2 ECD and streptavidin-coated paramagnetic beads as described (Hawkin *et al.* (1992) *J. Mol. Biol.* 226, 889-896) with some modifications. To prepare biotinylated antigen, 0.2 ml c-erbB-2 ECD (1 mg/ml) was incubated with 5 mM NHS-LC-Biotin (Pierce) overnight at 4°C and then purified on a presto desalting column. For each round of selection, 1 ml of phage (approximately 10<sup>13</sup> t.u.) were mixed with 1 ml PBS containing 4% skimmed milk powder, 0.05% Tween 20, and biotinylated c-erbB-2 ECD. Affinity-driven selections were performed by decreasing the amount of biotinylated c-erbB-2 ECD used for selection. Two selection schemes were used.

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In selection scheme 1 (S1) antigen concentrations of 10nM, 50 nM, 10 nM, and 1 nM were used for selection rounds 1, 2, 3, and 4 respectively. In selection scheme 2 (S2) antigen concentrations of 40 nM, 1 nM, 100 pM, and 10 pM were used for selection rounds 1, 2, 3, and 4 respectively. The mixture of phage and antigen was gently rotated on an under-and-over-turntable for 1 hour at room temperature. To capture phage binding biotinylated antigen, streptavidin coated M280 magnetic beads (Dynabeads, Dynal) were blocked with 2% MPBS for 1 h at 37°C, and then added to the mixture of phage and antigen. In S1, 200  $\mu$ l (round 1), 100  $\mu$ l (round 2) or 50  $\mu$ l (rounds 3 and 4) of beads were incubated with the phage-antigen mixture for 15 min,

30

rotating on an under-and-over-turntable at room temperature. In S2, 100  $\mu$ l (round 1) or 50  $\mu$ l (rounds 2, 3, and 4) of beads were incubated with the phage-antigen mixture for 15 min (round 1), 10 min (round 2), or 5 min (rounds 3 and 4). After capture of phage, Dynabeads were washed a total of 10 times (3 x PBS containing 0.05% Tween 20 (TPBS), 2 x TPBS containing 2% skimmed milk powder, x PBS, 1 x 2%MPBS, and 2 x PBS) using a Dynal magnetic particle concentrator. The Dynabeads were resuspended in 1 ml PBS, and 300  $\mu$ l were used to infect 10 ml log phase *E. coli* TG1 which were plated on TYE-AMP-GLU plates.

#### 10                    *Initial sFv characterization*

Initial analysis of chain shuffled sFv clones for binding tc-erbB-2 was performed by ELISA using bacterial supernatant containing expressed sFv. Expression of sFv (De Bellis and Schwartz (1990) *Nucleic Acids Res.* 18, 1311) was performed in 96 well microtitre plates as described (Marks *et al.* (1991), *supra*) with the following exception. After overnight growth and expression at 30°C, 50  $\mu$ l 0.5% Tween 20 was added to each well and the plates incubated for 4 h at 37°C with shaking to induce bacterial lysis and increase the concentration of sFv in the bacterial supernatant. For selection performed on Immunotubes, ELISA plates (Falcon 3912) were incubated with c-erbB-ECD (2.5  $\mu$ g/ml) in PBS at 4°C overnight. For selections performed with biotinylated protein, Immunolon 4 plates (Dynatech) were incubated overnight at 4°C with Immunopure avidin (10  $\mu$ g/ml in PBS; Pierce). After washing 3 times with PBS to remove unbound avidin, wells were incubated with biotinylated c-erbB-2 ECD as in Example 1. In both cases, binding of sFv to c-erbB-2 ECD was detected with the mouse monoclonal antibody 9E10 (1  $\mu$ g/ml), which recognizes the C-terminal peptide tag (Munro and Pelham (1986), *Cell* 46, 291-300) and peroxidase-conjugated anti-mouse Fc antibody (Sigma), as described (Marks *et al.*, 1991, *supra*). Selected binders were further characterized by sequencing of the V<sub>H</sub> and V<sub>L</sub> genes (Sanger *et al.* (1977) *Proc. Natl. Acad. Sci. USA*, 74: 5463-5467). Sequence data has been deposited with the GenBank Data Library.

30                    Screening of sFv for relative affinity was performed essentially as described (Friguet *et al.* (1985) *J. Immunol. Meth.* 77: 305-319). Immunolon 4 ELISA plates (Dynatech) were coated with avidin in PBS (10  $\mu$ g/ml) at 4°C overnight. Biotinylated c-erbB-2 ECD (5  $\mu$ g/ml) was added to the wells and incubated for 30 min at

room temperature. Bacterial supernatant containing sFv was incubated with varying concentrations of c-erbB-2 (0 to 100 nM) at 4°C for 1 h. The amount of free sFv was then determined by transferring 100  $\mu$ l of each mixture into the wells of the previously prepared ELISA plate and incubating for 1h at 4°C. Binding of sFv was detected as  
5 under ELISA screening and the IC50 calculated as described (Friguet *et al.* (1985), *supra*)

Screening of sFv by dissociation rate constant ( $k_{off}$ ) was performed using real-time biospecific interaction analysis based on surface plasmon resonance (SPR) in a BIAcore (Pharmacia). Typically 24 ELISA positive clones from each of the final two  
10 rounds of selection were screened. A 10 ml culture of *E. coli* TG1 containing the appropriate phagemid was grown and expression of sFv induced with IPTG (De Bellis and Schwartz, 1990). Cultures were grown overnight at 25°C, sFv harvested from the periplasm (Breitling *et al.* (1991) *Gene* 104, 147-153), and the periplasmic fraction dialyzed for 24 h against HEPES buffered saline (10 mM HEPES, 150 mM NaCl, pH 7.4,  
15 HBS). In a BIAcore flow cell, approximately 1400 resonance units (RU) of c-erbB-ECD (25  $\mu$ g/ml) in 10 mM acetate buffer pH 4.5 were coupled to a CM5 sensor chip (Johnsson *et al.* (1991) *Anal. Biochem.* 198, 268-277). Association and dissociation of undiluted sFv in the periplasmic fraction was measured under a constant flow of 5  $\mu$ l/min. An apparent dissociation rate constant ( $k_{off}$ ) was determined from the  
20 dissociation part of the sensorgram for each sFv analyzed (Karlsson *et al.* (1991) *J. Immunol. Methods* 145, 229-240). Typically 30 to 40 samples were measured during a single BIAcore run, with C6.5 periplasmic preparations analyzed as the first and final samples to ensure stability during the run. The flow cell was regenerated between samples using 2.6 M MgCl<sub>2</sub> in 10 mM glycine, pH 9.5 without significant change in the  
25 sensorgram baseline after analysis of more than 100 samples.

#### *Subcloning, expression and purification of Single-chain Fv.*

To facilitate purification, shuffled sFv genes were subcloned (Example 1) into the expression vector pUC11Sfi-NotmycHis, which results in the addition of a  
30 hexa-histidine tag at the C-terminal end of the sFv. 200 ml cultures of *E. coli* TG1 harboring one of the C6.5 mutant phagemids were grown, expression of sFv induced with IPTG (De Bellis and Schwartz (1990), *supra*) and the culture grown at 25°C overnight. Single-chain Fv was harvested from the periplasm (Breitling *et al.* (1991),

*supra*) dialyzed overnight at 4°C against 8 L of IMAC loading buffer (50 mM sodium phosphate, pH 7.5, 500 mM NaCl, 20 mM imidazole) and then filtered through a 0.2 micron filter.

5 Single-chain Fv was purified by immobilized metal affinity chromatography (IMAC) (Hochuli *et al.* (1988) *Bio/Technology*, 6, 1321-1325) as described in Example 1. To remove dimeric and aggregated sFv, samples were concentrated to a volume < 1 ml in a Centricon 10 (Amicon) and fractionated on a Superdex 75 column using a running buffer of HBS. The purity of the final preparation was evaluated by assaying an aliquot by SDS-PAGE. Protein bands were detected by  
10 Coomassie staining. The concentration was determined spectrophotometrically assuming an  $A_{280}$  nm of 1.0 corresponds to an sFv concentration of 0.7 mg/ml.

#### *Measurement of affinity, kinetics, and cell surface retention*

15 The  $K_d$  of light chain shuffled C6.5 mutants isolated from phage selection using Immuntubes (Nunc) were determined by Scatchard analysis. All assays were performed in triplicate. Briefly, 50 mg of radioiodinated sFv was added to  $5 \times 10^6$  SK-OV-cells in the presence of increasing concentrations of unlabeled sFv from the same preparation. After a 30 minute incubation at 20°C, the samples were washed with PBS at 4°C and centrifuged at 500g. The amount of labeled sFv bound to the cells was  
20 determined by counting the pellets in a gamma counter and the  $K_a$  and  $K_d$  were calculated using the EBDA program (V 2.0, G.A. McPherson, 1983). The  $K_d$  of all the other isolated sFv were determined using surface plasmon resonance in a BIAcore (Pharmacia). In a BIAcore flow cell, approximately 1400 resonance units (RU) of c-erbB-2 ECD (25  $\mu$ g/ml in 10 mM sodium acetate, pH 4.5) was coupled to a CM5 sensor chip (Johnsson  
25 *et al.* (1991), *supra*). Association and dissociation-rates were measured under continuous flow of 5 ml/min using a concentration range from 50 to 800 nM. Rate constant  $k_{on}$  was determined from a plot of  $(1/(dR/dt))/t$  vs concentration (Karlsson *et al.* (1991), *supra*). Rate constant  $k_{off}$  was determined from the dissociation part of the sensorgram at the highest concentration of sFv analyzed. Cell surface retention of C6.5 and C6L1 was  
30 determined as described in Example 1.

### *Modeling of location of mutations*

The location of mutations in shuffled sFv was modeled on the structure of the Fab KOL (Marquart *et al.* (1980) *J. Mol. Biol.* 141, 369-391) using MacImdad v5.0 (Molecular Applications Group, Palo Alto, CA) running on an Apple MacIntosh Quadra 650.

## **Results**

### *Construction of shuffled phage antibody libraries*

To facilitate heavy chain shuffling, libraries were constructed in pHEN-1 (Hoogenboom *et al.* (1991), *supra*) containing human V<sub>H</sub> gene repertoires (FR1 to FR3) and cloning sites for inserting the V<sub>H</sub> CDR3FR4, single chain linker, and light chain gene from a binding sFv as a BssHII-NotI fragment. Three heavy chain shuffling libraries were created (pHEN-1-V<sub>H</sub>1rep, pHEN-1-V<sub>H</sub>3rep, and pHEN-1-V<sub>H</sub>5rep), each enriched for V<sub>H</sub>1, V<sub>H</sub>3, or V<sub>H</sub>5 genes by using PCR primers designed to anneal to the consensus sequence of the 3' end of V<sub>H</sub>1, V<sub>H</sub>3, or V<sub>H</sub>FR3 (Tomlinson *et al.* (1992), *supra*). These primers also introduced a BssHII site at the end of FR3, without changing the amino acid sequence typically observed at these residues. Libraries of 5.0 x 10<sup>5</sup> clones for pHEN-1-V<sub>H</sub>1rep, 1.0 x 10<sup>6</sup> clones for pHEN-1-V<sub>H</sub>3rep and 1.5 x 10<sup>6</sup> clones for pHEN-1-V<sub>H</sub>5rep were obtained. Analysis of 50 clones from each library indicated that greater than 80% of the clones had inserts, and the libraries were diverse as shown by the BstNI restriction pattern (Marks *et al.* (1991), *supra*). Three heavy chain shuffled libraries were made by cloning the C6.5 V<sub>H</sub> CDR3, FR4, linker, and light chain genes into the previously created V<sub>H</sub>1, V<sub>H</sub>3, or V<sub>H</sub>5 repertoire using the BssHII and NotI restriction sites. After transformation, libraries of 1.0-2.0 x 10<sup>6</sup> clones were obtained. PCR screening revealed that 100% of clones analyzed had full length insert and diverse BstNI restriction pattern. Prior to selection, 20/92 clones selected at random from the V<sub>H</sub>5 library expressed sFv which bound c-erbB-2. 0/92 clones selected at random from the V<sub>H</sub>1 or V<sub>H</sub> repertoire expressed sFv which bound c-erbB-2.

To facilitate light chain shuffling, a library was constructed in pHEN-1 containing human V<sub>k</sub> and V<sub>l</sub> gene repertoires, single chain linker DNA, and cloning sites for inserting the V<sub>H</sub> gene from binding sFv as an NcoI-XhoI fragment. The resulting library (pHEN-1-V<sub>l</sub>rep) consisted of 4.5 x 10<sup>6</sup> clones. PCR screening revealed that 95% of clones analyzed had full length insert and a diverse BstNI restriction pattern. A light

chain shuffled library was made by cloning the C6.5 V<sub>H</sub> gene into pHEN-1-V<sub>L</sub>rep. After transformation a library of 2.0 x 10<sup>6</sup> clones was obtained. PCR screening revealed that 100% of clones analyzed had full length insert and a diverse BstNI restriction pattern. Prior to selection, 0/92 clones selected at random expressed sFv which bound c-erbB-2.

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*Isolation and characterization of higher affinity light chain shuffled scF*

In a first approach to increase affinity, c-erbB-2 ECD coated polystyrene tubes were used for selecting the light chain shuffled library. Phage were subjected to three rounds of the rescue-selection-infection cycle. One hundred and eighty clones from the 2nd and the 3rd round of selection were analyzed for binding to recombinant c-erbB-2 ECD by ELISA. After the 3rd round of selection, greater than 50% of the clones were positive by ELISA (Table 8).

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Table 8. Frequency of binding sFv and percent of binding sFv with slower k<sub>off</sub> than C6.5. binding was determined by ELISA. Rate constant k<sub>off</sub> was determined by BIAcore on unpurified sFv in bacterial periplasm.

Library and method of selection	ELISA			sFv with slower k <sub>off</sub> than C6.5 (parental sFv)		
	Round of Selection			Round of Selection		
	2	3	4	2	3	4
<b>V<sub>L</sub>-shuffling, selected on:</b>						
antigen coated immunotubes	41/180	97/180	ND	ND	ND	ND
soluble antigen (rd 1, 100 nM; rd 2, 50 nM; rd 3 10 nM; rd 4, 1 nM).	74/90	22/90	13/90	ND	0%	42%
soluble antigen (rd 1, 40 nM; rd 2, 1 nM; rd 3, 0.1 nM; rd 4, 0.01 nM)	ND	65/90	62/90	ND	25%	84%
<b>V<sub>H</sub>-shuffling, selected on:</b>						
soluble antigen; (rd 1, 100 nM; rd 2, 50 nM; rd 3, 10 nM; rd 4, 1 nM)	ND	43/90	56/90	ND	0%	0%
soluble antigen (rd 1, 40 nM; rd 2, 1 nM; rd 3, 0.1 nM; rd 4, 0.01 nM)	ND	90/90	82/90	ND	0%	12%

rd=round, ND= not determined, nM=1.0 x 10<sup>-9</sup> M

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Table 9.  $IC_{50}$  and  $K_d$  of C6.5sFv and 4 chain shuffled mutant sFvs.  $IC_{50}$  was determined by competition ELISA and  $K_d$  by Scatchard after radioiodination.

sFv	$IC_{50}$ (M)	$K_d$ (M)
C6.5	$2.0 \times 10^{-8}$	$2.0 \times 10^{-8}$
C6VLB	$1.0 \times 10^{-8}$	$3.0 \times 10^{-8}$
C6VLD	$5.8 \times 10^{-9}$	$2.6 \times 10^{-8}$
C6VLE	$2.8 \times 10^{-9}$	$7.1 \times 10^{-8}$
C6VLF	$7.5 \times 10^{-9}$	$7.9 \times 10^{-8}$

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Table 10. Deduced protein sequences of light chain variable region genes of C6.5 and chain shuffled mutants.

	Framework 1	CDR1	Framework 2	CDR2	Framework 3	CDR3	Framework 4
	10 20 30	35 40 50	55 60 70	75 80 90	95 100		
C6.5	QSVLTQPPSVSAAPGAKVTISC	SGSSNIGMTVS	<u>MYOOLPGTAPKLLIY</u>	GHTMRPA	GVPDFSGSKSGTSASLAI	SGRSEDEADYYC	<u>AAADDLSLG</u> <u>WV</u> <u>EGGGIKLIVLG</u>
Light chain shuffled mutants selected on polystyrene adsorbed antigen:							
C6VLB	-----	-----	-----	SDNQ--S	-----	-----	-----H
C6VLD	-----	-TW-----	-----	TNDQ--S	-----	-----	-----M--H
C6VLE	-----	A-----S-R-----	-----	-W RNNQ--S	-----	-----	-----S--N-----
C6VLF	-----	M-----	-----	H DNKK--S -I-----	-----	-----	-----LQ-D-----N-----
Light chain shuffled mutant selected on biotinylated antigen:							
C6L1	-----G-----W-----	-----	-----	DNKK--S	-----	-----	-----L-----

CDR, complementarity determining region; dashes indicate sequence identity. Numbering is according to Kabat *et al.*, 1987, *supra*. Underlined residues are those that form the  $\beta$ -sheet interface that packs on the VH domain (Chothia *et al.*, 1985 *supra*).

Positive clones were ranked by  $IC_{50}$  as determined by competition ELISA (Table 9). Sixteen sFv with  $IC_{50}$ s less than the  $IC_{50}$  of the parental sfv were sequenced and four unique DNA sequences identified (Table 10). These clones were purified by IMAC after subcloning into PUC119SFI/NotmycHis, and the affinity determined by  
5 Scatchard analysis.

Despite their lower  $IC_{50}$ s, none of these 4 sfv had a higher affinity for c-erbB-2 (Table 9). Gel filtration analysis of the four purified sfv demonstrated the presence of two species, with size consistent for monomeric and dimeric sfv. In contrast, the parental sFv existed only as monomer.

10 As a result of these observations, it was hypothesized that selection on immobilized antigen favored the isolation of lower affinity dimeric sFv which could achieve a higher apparent affinity due to avidity. In addition, determination of  $IC_{50}$  by inhibition ELISA using native sFv in periplasm did not successfully screen for sFv of higher affinity. To avoid the selection of lower affinity dimeric sFv, subsequent  
15 selections were performed in solution by incubating the phage with biotinylated c-erbB-2 ECD, followed by capture on streptavidin coated magnetic beads. To select phage on the basis of affinity, the antigen concentration was reduced each round of selection to below the range of the desired sFv  $K_d$  (Hawkins *et al.* (1992), *supra*). To screen ELISA positive sFv for improved binding to c-erbB-2, a BIAcore was used. Periplasm  
20 preparations containing unpurified native sFv can be applied directly to a c-erbB-2 coated BIAcore flow cell, and the  $k_{off}$  determined from the dissociation portion of the sensorgram. This permitted ranking the chain shuffled clones by  $k_{off}$ . Moreover, by plotting  $\ln(Rn/R_0)$  vs  $t$ , the presence of multiple  $k_{off}$  can be detected, indicating the presence of mixtures of monomeric and dimeric sFv. This strategy of selecting on  
25 antigen in solution, followed by BIAcore screening of ELISA positive sFv, was used to isolate higher affinity chain shuffle mutants.

The light chain shuffled library was subjected to four rounds of selection on decreasing soluble antigen concentration (100 nM, 50 nM, 10 nM, and, 1 nM). In a separate set of experiments, the 4 rounds of selection were performed using 40 nM, 1  
30 nM, 0.1 nM, and 0.01 nM antigen concentration. Using the higher set of antigen concentrations for selection, 13/90 clones were positive for c-erbB-binding by ELISA after the 4th round of selection. In the BIAcore, 42% of these clones had a slower  $k_{off}$  than the parental sFv. Using the lower set of antigen concentrations for selection, more

clones were positive for c-erbB-2 binding by ELISA (62/90) after the 4th round of selection, and 84% had a slower  $k_{\text{off}}$  than the parental sFv. Sequencing of the  $V_L$  gene of ten of these sFv revealed one unique sFv (C6L1) (Table 10). The  $V_L$  gene of C6L1 was derived from the same germline gene as the parental sFv, but had 9 amino acid substitutions. The C6L1 gene was subcloned and the sFv purified by IMAC and gel filtration. C6L1 sFv was monomeric as determined by gel filtration and had an affinity 6 times higher than parental (Table 11). The increased affinity was due to both a faster  $k_{\text{on}}$  and a slower  $k_{\text{off}}$  (Table 11). The slower  $k_{\text{off}}$  was associated with a three fold increase in the retention of sFv on the surface of SK-OV-3 cells (28% at 30 minutes for C6L1 compared to 10% at 3 minutes for the parental sFv).

**Table 11.** Affinities and binding kinetics of c-erbB-2 binding Single-chain Fv,  $K_d$ ,  $k_{\text{on}}$  and  $k_{\text{off}}$  were determined by surface plasmon resonance in a BIAcore. Combined Single-chain Fv result from combining the  $V_L$  of C6L1 with the  $V_H$  of either C6H1 or C6H2.

sFv source and clone name	$K_d$ (M)	$k_{\text{on}}$ ( $M^{-1}s^{-1}$ )	$k_{\text{off}}$ ( $M^{-1}s^{-1}$ )
Parental C6.5	$1.6 \times 10^{-8}$	$4.0 \times 10^{-5}$	$6.3 \times 10^{-3}$
Light Chain Shuffled C6L	$2.6 \times 10^{-9}$	$7.8 \times 10^{-5}$	$2.0 \times 10^{-3}$
Heavy Chain Shuffled C6H1	$5.9 \times 10^{-9}$	$1.1 \times 10^{-6}$	$6.2 \times 10^{-3}$
C6H2	$3.1 \times 10^{-9}$	$8.4 \times 10^{-5}$	$2.6 \times 10^{-3}$
Combined sFv C6H1L1	$1.5 \times 10^{-8}$	$4.1 \times 10^{-5}$	$6.2 \times 10^{-3}$
C6H2L1	$6.0 \times 10^{-9}$	$3.0 \times 10^{-5}$	$1.8 \times 10^{-3}$

#### *Isolation and characterization of higher affinity heavy chain shuffled scf.*

The  $V_H5$  heavy chain shuffled library was subjected to four rounds of selection on decreasing soluble antigen concentration (100 nM, 5 nM, 10 nM, and, 1 nM). In a separate set of experiments, the rounds of selection were performed using 40 nM, 1 nM, 0.1 nM, and 0.01 nM antigen concentration. Using the higher set of antigen concentrations for selection, 56/90 clones were positive for c-erbB-binding by ELISA after the 4th round of selection. None of these clones, however, had a slower  $k_{\text{off}}$  than the parental sFv. Using the lower set of antigen concentrations for selection, more clones were positive for c-erbB-2 binding by ELISA (82/90) after the 4th round of selection, and 12% had a slower  $k_{\text{off}}$  than the parental sFv. No binders were isolated

from either the V<sub>H</sub>1 or V<sub>H</sub>3 shuffled libraries. Sequencing of the V<sub>H</sub> gene of all slower k<sub>off</sub> clones revealed two unique sFv, C6H1 and C6H2 (Table 12). The V<sub>H</sub> genes of C6H1 and C6H2 were derived from the same germline gene as the parental sFv, but differed by 7 and 9 amino acids respectively. C6H1 also had a stop codon in the heavy chain CDR1 and was expressed as a PIII fusion due to read through, albeit at very low levels. The two sFv were subcloned and purified by IMAC and gel filtration. Both sFv were monomeric as determined by gel filtration C6H1 had 3 fold higher affinity for c-erbB-2 than C6.5 and C6H2 had 5 fold higher affinity than C6.5 (Table 11). The increased affinity of C6H(5.9 x 10<sup>-9</sup> M) was due to a faster k<sub>on</sub>, whereas the increased affinity of C6H2 (3.1 x 10<sup>-9</sup> M) was due to both a faster k<sub>on</sub> and slower k<sub>off</sub> (Table 11).

#### *Location of mutations in chain shuffled scf*

Mutations in chain shuffled sFv were modeled on the Fv fragment of the immunoglobulin KOL (Marquart *et al.* (1980), *supra*) (Figures 2 and 3). KOL was selected as the model because it has a V<sub>λ</sub> gene derived from the same family as C6.5, and a V<sub>H</sub> gene with the same length CDR2. Mutations in higher affinity sFv were located both in surface residues at the antigen combining site, as well as residues located far from the binding site (Figure 2). Except for two conservative mutations in V<sub>H</sub> framework 3 (V89M and F91Y), no mutations were located in residues which form the two 5 stranded β-sheets that form the V<sub>H</sub>-V<sub>L</sub> interface (Chothia *et al.* (1985) *J. Mol. Biol.* 186, 651-663) (figure 2 and Tables 10 and 12). In contrast, all 4 light chain shuffled sFv which formed mixtures of monomer and dimer had mutations in residues which formed the β-sheet that packs on the V<sub>H</sub> domain (Table 4 and Figure 3).



*Affinities of sFv resulting from combining higher affinity V<sub>H</sub> and V<sub>L</sub> genes obtained by chain shuffling.*

In an attempt to further increase affinity, shuffled V<sub>H</sub> and V<sub>L</sub> genes from higher affinity sFv were combined into the same sFv. Combining the V<sub>L</sub> gene from C6L1 with the V<sub>H</sub> gene from C6H1 resulted in an sFv (C6H1L1) with lower affinity than either C6L1 or C6H2 (Table 11). No additional reduction in k<sub>off</sub> was achieved, and the k<sub>on</sub> was reduced approximately 2 fold. Similarly, combining the V<sub>L</sub> gene from C6L1 with the V<sub>H</sub> gene from C6H2 resulted in an sFv (C6H2L1) with lower affinity than C6L1 or C6H2 (Table 11). No additional reduction in k<sub>off</sub> was achieved, and the k<sub>on</sub> was reduced approximately 2 fold. Thus, in both instances, combining the independently isolated higher affinity V<sub>H</sub> and V<sub>L</sub> genes had a negative effect on affinity.

**Example 3**

**Production of Higher Affinity Mutants**

In order to prepare higher affinity mutants derived from C6.5 part of the light chain and heavy chain CDR3 were sequentially randomized. The C6.5 VL CDR3 was modified by randomizing the sequence AAWDDSLSG. The heavy chain CDR3 domain was randomized. The variable heavy chain CDR3 was randomized 4 amino acids at a time: In other words, the CDR3 sequence of HDVGYCSSSNCAKWPEYFQH was modified by randomizing SSSN (library B), DVGY (library A), AKPE (library C) and YFQH (library D) SSSN, AKPE and YFQH respectively as described below.

**I. Materials and Methods**

*Construction of phage antibody libraries*

As explained above, mutant sFv phage antibody libraries were constructed based on the sequence of C6.5, a human sFv isolated from a non-immune phage antibody library which binds to the tumor antigen c-erbB-2 with a K<sub>d</sub> of 1.6 x 10<sup>-8</sup> M (see Example 1). For construction of a library containing V<sub>L</sub> CDR3 mutants, an oligonucleotide (VL1; Table 18) was designed which partially randomized nine amino acid residues located in V<sub>L</sub> CDR3 (Table 4, above). For the nine amino acids randomized, the ratio of nucleotides was chosen so that the frequency of wild-type (wt) amino acid was 49%.

To create the library, C6.5 sFv DNA (10 ng) was amplified by PCR in 50  $\mu$ l reactions containing 25 pmol LMB3 (Marks *et al.*, 1991 *J. Mol. Biol.* 222: 581-597), 25 pmol VL1, 250  $\mu$ M dNTPs, 1.5 mM MgCl<sub>2</sub>, and 1  $\mu$ l (5 units) Taq DNA polymerase (Promega) in the manufacturers buffer. The reaction mixture was subjected to 30 cycles of amplification (94°C for 30 s, 50°C for 30 s and 72°C for 1 min) using a Hybaid OmniGene cycler.

To introduce a NotI restriction site at the 3' end of the sFv gene repertoire, the PCR fragment (850 bp) was gel purified and reamplified using the primers LMB3 and VL2 (Table 18). The PCR product was purified, digested with SfiI and NotI, and ligated into pCANTAB5E (Pharmacia) digested with SfiI and NotI.

Ligation mixtures were purified as previously described above and aliquots electroporated (Dower *et al.* (1988) *Nucleic Acids Res.*, 16: 6127-45) into 50  $\mu$ l *E. coli* TG1 (Gibson (1984) Studies on the Epstein-Barr virus genome. PhD thesis, University of Cambridge). Cells were grown in 1 ml SOC (Sambrook *et al.*, (1990) *supra.*) for 30 min and then plated on TYE (Miller, J. H. (1972) *Experiments in Molecular Genetics*, Cold Springs Harbor Lab Press, Cold Springs Harbor, New York.) media containing 100  $\mu$ g ampicillin/ml and 1% (w/v) glucose (TYE-AMP-Glu). Colonies were scraped off the plates into 5 ml of 2 x TY broth (Miller (1972) *supra.*) containing 100  $\mu$ g ampicillin/ml, 1% glucose (2xTY-AMP-Glu) and 15% (v/v) glycerol for storage at -70°C. The cloning efficiency and diversity of libraries was determined by PCR screening (Gussow & Clackson (1989) *Nucleic Acids Res.* 17) exactly as described in (Marks *et al.*, (1991) *supra.*) and by DNA sequencing (Sanger *et al.* (1977) *Proc. Natl. Acad. Sci. USA*, 74: 5463-7). The mutant phage antibody library was designated C6VLCDR3.

Four libraries of V<sub>H</sub> CDR3 mutants were constructed. For construction of each V<sub>H</sub> CDR3 library, oligonucleotides (VHA, VHB, VHC, and VHD; Table 18) were designed which completely randomized four amino acid residues located in V<sub>H</sub> CDR3 (amino acid residues 96 to 99, library A; residues 100a to 100d, library B; residues 100f, 100g, 100i, and 100j, library C; and residues 100k to H102, library D; Table 13). To create the libraries, DNA encoding the V<sub>H</sub> gene of C6.5 sFv DNA (10 ng) was amplified by PCR in 50  $\mu$ l reactions containing 25 pmol LMB3 (Marks *et al.*, 1991) and 25 pmol of either VHA, VHB, VHC, or VHD exactly as described above. The resulting PCR fragments were designated VHA1, VHB1, VHC1, and VHD1, based on the

mutagenic oligonucleotide used for amplification. In four separate PCR reactions, DNA encoding the light chain, sFv linker, V<sub>H</sub> framework 4 (FR4), and a portion of V<sub>H</sub> CDR3 of C6ML3-9 was amplified by PCR as described above using the primers C6hisnot and either RVHA, RVHB, RVHC, or RVHD (Table 18).

5                    These amplifications yielded PCR fragments VHA2, VHB2, VHC2, and VHD2. The 5' end of primers RVHA, RVHB, RVHC, and RVHD were designed to be complementary to the 5' ends of primers VHA, VHB, VHC, and VHD respectively. This complementarity permits joining of the VH1 and VH2 PCR fragments together to create a full length sFv gene repertoire using splicing by overlap extension. To create  
10                    the mutant sFv gene repertoires, 200 ng of each PCR fragment (VHA1 and VHA2, VHB1 and VHB2, VHC1 and VHC2, or VHD1 and VHD2) were combined in 50 ml PCR reaction mixtures (as described above) and cycled seven times to join the fragments (94°C for 30s, 60°C for 5s, 40°C for 5s (RAMP: 5s), 72°C for 1 min). After seven  
15                    cycles, outer primers LMB3 and C6hisnot were added and the mixtures amplified for 30 cycles (94°C for 30 s, 50°C for 30 s, 72°C for 1 min). The PCR products were purified as described above, digested with SfiI and NotI, and separately ligated into pCANTAB5E (Pharmacia) digested with SfiI and NotI. The four ligation mixtures were purified as described above and electroporated into 50 µl *E. coli* TG1. Transformed cells were grown and plated, and libraries characterized and stored, as described above.  
20                    The mutant phage antibody libraries were designated C6VHCDR3A, C6VHCDR3B, C6VHCDR3C, and C6VHCDR3D.

*Preparation of phage and selection of phage antibody libraries.*

25                    Preparation of phage for selection was performed exactly as described in Examples 1 and 2.. Phage particles were purified and concentrated by two PEG-precipitations (Sambrook *et al.*, 1990), resuspended in 5 ml phosphate buffered saline (25 mM NaH<sub>2</sub>PO<sub>4</sub>, 125 mM NaCl, pH 7.0, PBS) and filtered through a 0.45 µ filter. All libraries were selected using biotinylated c-erbB-2 ECD and streptavidin-coated paramagnetic beads M280 (Dyna) as described above. For selection  
30                    of the C6VLCDR3 library, c-erbB-2 ECD concentrations of 4.0 x 10<sup>-8</sup> M, 1.0 x 10<sup>-9</sup> M, 1.0 x 10<sup>-10</sup> M, and 1.0 x 10<sup>-11</sup> M were used for selection rounds 1, 2, 3, and 4 respectively. The mixture of phage and antigen was gently rotated for 1 h at room temperature and phage bound to biotinylated antigen captured using 100 µl (round 1) or

50  $\mu$ l (rounds 2, 3, and 4) of streptavidin-coated M280 magnetic beads. After capture of phage, Dynabeads were washed a total of ten times (three times in PBS containing 0.05% Tween 20 (TPBS), twice in TPBS containing 2% skimmed milk powder (2%MPBS), twice in PBS, once in 2%MPBS, and twice in PBS) using a Dynal magnetic particle concentrator. The Dynabeads were resuspended in 1 ml PBS, and 300  $\mu$ l were used to infect 10 ml log phase *E. coli* TG1 which were plated on TYE-AMP-Glu plates. For selection of the C6VHCDR3 libraries, c-erbB-2 ECD concentrations of  $5.0 \times 10^{-9}$  M,  $5.0 \times 10^{-11}$  M,  $5.0 \times 10^{-12}$  M, and  $5.0 \times 10^{-13}$  M were used for selection rounds 1, 2, 3, and 4 respectively and the phage captured by incubating with 50  $\mu$ l of Dynabeads for 5 min.

The washing protocol was altered to select for sFv with the lowest  $k_{off}$  (Hawkins *et al.* (1992) *J. Mol. Biol.* 226: 889-896). Dynabeads with bound phage were initially subjected to five rapid washes (4 x TPBS, 1 x MPBS) followed by six 30 min incubations in one of three washing buffer (2 x TPBS, 2 x MPBS, 2 x PBS) containing  $1.0 \times 10^{-7}$  M c-erbB-2 ECD. Bound phage were eluted from the Dynabeads by sequential incubation with 100  $\mu$ l of 4 M  $MgCl_2$  for 15 min followed by 100  $\mu$ l of 100 mM HCl for 5 min. Eluates were combined and neutralized with 1.5 ml of 1 M Tris HCl, pH 7.5 and one third of the eluate used to infect log phase *E. coli* TG1.

#### *Initial sFv characterization.*

Initial analysis of selected sFv clones for binding to c-erbB-2 ECD was determined by phage ELISA. To prepare phage for ELISA, single ampicillin resistant colonies were transferred into microtitre plate wells containing 100  $\mu$ l 2xTY-AMP-0.1% glucose and grown for three hours at 37°C to an  $A_{600}$  of approximately 0.5. VCSM13 helper phage ( $2.5 \times 10^8$  phage) were added to each well, and the cells incubated for 1 hour at 37°C.

Kanamycin was then added to each well to a final concentration of 25  $\mu$ g/ml and the bacteria grown overnight at 37°C. Supernatants containing phage were used for ELISA. For ELISA, Immunolon 4 plates (Dynatech) were incubated overnight at 4°C with ImmunoPure avidin (10  $\mu$ g/ml in PBS; Pierce). After washing three times with PBS to remove unbound avidin, wells were incubated with biotinylated c-erbB-2 ECD as described above.

Binding of sFv phage to c-erbB-2 ECD was detected with peroxidase-conjugated anti-M13 antibody (Pharmacia) and ABTS (Sigma) as substrate. Selected binders were further characterized by DNA sequencing of the  $V_H$  and  $V_L$  genes.

Ranking of sFv by  $k_{off}$  was performed using SPR in a BIAcore as described above. Briefly, 10 ml cultures of 24 ELISA positive clones from the third and fourth round of selection were grown to an  $A_{600}$  of approximately 0.8, sFv expression induced (De Bellis *et al.* (1990). *Nucleic Acids Res.*, 18: 1311) and the culture grown overnight at 25°C. Single-chain Fv were harvested from the periplasm (Breitling *et al.* (1991) *Gene*, 104: 147-153), and the periplasmic fraction dialyzed for 48 h against hepes buffered saline (10 mM hepes, 150 mM NaCl, pH 7.4, HBS). In a BIAcore flow cell, approximately 1400 resonance units (RU) of c-erbB-2 ECD were coupled to a CM5 sensor chip using NHS-EDC chemistry (Johnsson *et al.* (1991) *Anal. Biochem.* 198: 268-277). Association and dissociation of undiluted sFv in the periplasmic fraction were measured under a constant flow of 5  $\mu$ l/min and HBS as running buffer. An apparent  $k_{off}$  was determined from the dissociation part of the sensorgram for each sFv analyzed (Karlsson *et al.* (1993) *J. Immunol. Meth.* 166: 75-84). The flow cell was regenerated between samples using sequential injections of 4 M  $MgCl_2$  and 100 mM triethylamine without significant change in the sensorgram baseline after analysis of more than 100 samples.

#### *Subcloning, expression and purification of sFv.*

To facilitate purification of sFv selected from the C6VLCDR3 library, the sFv genes were subcloned into the expression vector pUC119 Sfi-NotmycHis, which results in the addition of a hexa-histidine tag at the C-terminal end of the sFv. The sFv selected from the C6VHCDR3 library already have a C-terminal hexa-histidine tag and therefore could be purified without subcloning. 500 ml cultures of *E. coli* TG1 harboring one of the C6.5 mutant phagemids were grown, expression of sFv induced (De Bellis *et al.* (1990) *supra.*), and the culture grown at 25°C overnight. Single-chain Fv were harvested from the periplasm (Breitling *et al.* (1991) *supra.*), dialyzed overnight at 4°C against 8 L of IMAC loading buffer (50 mM sodium phosphate, pH 7.5, 500 mM NaCl, 20 mM imidazole) and then filtered through a 0.2 micron filter. Single-chain Fv was purified by IMAC (Hochuli *et al.* (1988) *Bio/Technology*, 6: 1321-1325) as described above.

To separate monomeric, dimeric and aggregated sFv, samples were concentrated to a volume < 1 ml in a Centricon 10 (Amicon) and fractionated on a Superdex 75 column using a running buffer of HBS. The purity of the final preparation was evaluated by assaying an aliquot by SDS-PAGE. Protein bands were detected by  
5 Coomassie staining. The concentration was determined spectrophotometrically, assuming an  $A_{280}$  nm of 1.0 corresponds to an sFv concentration of 0.7 mg/ml.

*Measurement of affinity and binding kinetics.*

The  $K_d$  of sFv were determined using SPR in a BIAcore. In a BIAcore  
10 flow cell, approximately 1400 RU of c-erbB-2 ECD (90 kDa, McCartney *et al.* (1995) *Protein Eng.* 8: 301-314) were coupled to a CM5 sensor chip (Johnsson *et al.* (1991) *supra.*). Association rates were measured under continuous flow of 5 ml/min using concentrations ranging from  $5.0 \times 10^{-8}$  to  $8.0 \times 10^{-7}$  M. Rate constant  $k_{on}$  was determined from a plot of  $(\ln(dR/dt))/t$  vs concentration (Karlsson *et al.*, 1991).

15 To verify that differences in  $k_{on}$  were not due to differences in immunoreactivity, the relative concentrations of functional sFv was determined using SPR in a BIAcore (Karlsson *et al.* (1993) *supra.*). Briefly, 4000 RU of c-erbB-2 ECD were coupled to a CM5 sensor chip and the rate of binding of C6.5 (RU/s) determined under a constant flow of 30 ml/min. Over the concentration range of  $1.0 \times 10^{-9}$  M to  $1.0$   
20  $\times 10^{-7}$  M, the rate of binding was proportional to the log of the sFv concentration. Purified sFv were diluted to the same concentration ( $1.0 \times 10^{-8}$  M and  $2.0 \times 10^{-8}$  M) as determined by  $A_{280}$ . The rate of binding to c-erbB-2 ECD was measured and used to calculate the concentration based on the standard curve constructed from C6.5. Dissociation rates were measured using a constant flow of 25  $\mu$ l/min and a sFv  
25 concentration of  $1.0 \times 10^{-6}$  M.  $k_{off}$  was determined during the first 2 min of dissociation for sFv mutated in  $V_L$  CDR3 (Karlsson *et al.* (1991) *supra.*) and during the first 15 to 60 min for clones with  $k_{off}$  below  $5 \times 10^{-4}$  s $^{-1}$  (sFv mutated in  $V_H$  CDR3 and combined sFv). To exclude rebinding,  $k_{off}$  was determined in the presence and absence of  $5.0 \times 10^{-7}$  M c-erbB-2 ECD as described above in Examples 1 and 2.

30

*Cell surface retention assay.*

The cell surface retention of selected sFv was determined on live SK-OV-3 cells using a fluorescence activated cell sorter (FACS). Purified sFv were labeled with

NHS-LC-Biotin (Pierce) using the manufacturers instructions. The concentration of immunoreactive biotinylated sFv was calculated using SPR as described above. The efficiency of biotinylation was also determined in a BIAcore using a flow cell to which 5000 RU of streptavidin was coupled. The total responses after association were compared between samples and concentrations of sFv were adjusted using the results obtained from the BIAcore. For the assay, aliquots of SK-OV-3 cells ( $1.2 \times 10^7$  c-erbB-2 positive cells) were incubated with 14  $\mu$ g biotinylated sFv in a total volume of 0.5 ml (1  $\mu$ M sFv) FACS buffer (PBS containing 1% BSA and 0.1% NaN<sub>3</sub>) for 1 h at 37°C. Cells were washed twice with 10 ml FACS buffer (4°C) and resuspended in 12 ml FACS buffer and further incubated at 37°C. Aliquots of cells (0.5 ml from 12 ml containing  $5 \times 10^5$  cells) were taken after 5 min, every 15 min for the first hour and after two hours repeating the wash and resuspension cycle. Washed cell aliquots were fixed with 1% paraformaldehyde, washed twice with FACS buffer, and incubated for 15 min at 4°C with a 1:800 dilution of phycoerythrine-labeled streptavidin (Pierce).

Fluorescence was measured by FACS and the percent retained fluorescence on the cell surface plotted versus the time points. Single-chain Fv used for the cell surface retention assay were C6.5 ( $K_d = 1.6 \times 10^{-8}$  M), C6ML3-9 ( $K_d = 1.0 \times 10^{-9}$  M), C6MH3-B1 ( $K_d = 1.2 \times 10^{-10}$  M), and the anti-digoxin sFv 26-10 (Huston *et al.* (1988) *Proc. Natl. Acad. Sci. USA*, 85: 5879-83) as negative control.

#### *High resolution functional scan of C6.5 V<sub>H</sub> CDR3.*

A high resolution functional scan of the C6.5 V<sub>H</sub> CDR3 was performed by individually mutating residues 95-99, 100a-100d, and 100g-102 to alanine. The pair of cysteine residues (100 and 100e) were simultaneously mutated to serine. Residue 100f (alanine) was not studied. Mutations were introduced by oligonucleotide directed mutagenesis using the method of Kunkel *et al.* (1987) *Meth. Enzymol.*, 154: 367-82.

Insertion of the correct mutation was verified by DNA sequencing, and sFv was expressed (De Bellis *et al.* (1990) *supra.*; Breitling *et al.* (1991) *supra.*) and purified by IMAC (Hochuli *et al.* (1988) *supra.*). Affinities were determined by SPR as described above and compared to C6.5 sFv.

#### *Modeling of location of mutations.*

The location of mutations in mutated sFv was modeled on the structure of the Fab KOL (Marquart et al. (1980) *J. Mol. Biol.*, 141: 369-391) using the program O (Jones et al. (1991). *Acta Cryst.*, A47: 110-119) on a Silicon Graphics workstation.

## 5 **II. Results**

### 1) Mutation of C6.5 sFv V<sub>L</sub> CDR3

#### *Library construction and selection.*

10 As explained above, 9 amino acids in V<sub>L</sub> CDR3 were partially randomized by synthesizing a “doped” oligonucleotide in which the wild-type nucleotide occurred with a frequency of 49%. After transformation, a library of  $1.0 \times 10^7$  clones was obtained. The mutant phage antibody library was designated C6VLCDR3.

15 Polymerase chain reaction (PCR) screening revealed that 100% of clones analyzed had full length insert and diversity was confirmed by sequencing the V<sub>L</sub> CDR3 of ten clones from the unselected library. Prior to selection, 5/92 clones selected at random expressed sFv which bound c-erbB-2 ECD by enzyme linked immunosorbent assay (ELISA).

20 The C6VLCDR3 library was subjected to four rounds of selection using decreasing concentrations of biotinylated c-erbB-2 ECD. A relatively high antigen concentration ( $4.0 \times 10^{-8}$  M) was used for the first round to capture rare or poorly expressed phage antibodies. The concentration was decreased 40 fold for the second round ( $1.0 \times 10^{-9}$  M), and decreased a further tenfold each of the subsequent two rounds ( $1.0 \times 10^{-10}$  M, 3rd round;  $1.0 \times 10^{-11}$  M, 4th round). After each round of selection, the concentration of binding phage in the polyclonal phage preparation was determined by measuring the rate of binding of polyclonal phage to c-erbB-2 ECD under mass transport limited conditions using surface plasmon resonance (SPR) in a BIAcore. The results 25 were used to guide the antigen concentration for the subsequent round of selection. After both the third and fourth rounds of selection, 92/92 clones bound c-erbB-2 ECD by ELISA.

#### 30 *Characterization of mutant sFv.*

To identify sFv with a lower  $K_d$  than wild-type sFv, apparent  $k_{off}$  was determined by SPR in a BIAcore on unpurified native sFv in bacterial periplasm.

Twenty-four sFv from the third and fourth rounds of selection were ranked by  $k_{off}$ . After

the third round of selection, 80% of sFv had a lower  $k_{off}$  than wt and after four rounds, 100% of sFv had a lower  $k_{off}$  than wild-type sFv. The twelve sFv with the lowest  $k_{off}$  from each of these rounds of selection were sequenced and each unique sFv gene was subcloned for purification. Single-chain Fv were purified by immobilized metal affinity chromatography (IMAC), followed by gel filtration to remove any dimeric or aggregated sFv.

The  $k_{on}$ , and  $k_{off}$  were determined by BIAcore, and the  $K_d$  calculated. After the third round of selection, seven unique sFv were identified, all with higher affinity than wild-type sFv. Single-chain Fv had on average 1.8 amino acid substitutions/sFv, with a single substitution at residue 92 the most frequently observed mutation. These single amino acid substitutions would have occurred with a frequency of 1/12,000 in the original library, assuming equal nucleotide coupling efficiency. The average sFv affinity was  $3.6 \times 10^{-9}$  M (4.4 fold increase), with the highest affinity  $2.6 \times 10^{-9}$  M (sixfold increase).

After four rounds of selection, six sFv were identified, and none of these sequences were observed in the sFv sequenced from the third round. Single-chain Fv from the fourth round had on average 2.9 amino acid substitutions/sFv, with expected frequencies of between 1/590,000 and 1/24,000,000 in the original library. The average sFv affinity after the fourth round was  $1.9 \times 10^{-9}$  M (8.4 fold increase), with the highest affinity  $1.0 \times 10^{-9}$  M (16 fold increase). The results demonstrate the efficiency of the selection technique for isolating very rare high affinity clones from a library. Additional high affinity sFv (Table 14; C6ML3-14, -15, -19, -23, and -29) were isolated from the C6VLCADR3 library by using a different elution solution after capture of antigen bound phage.

25

*Location of mutations in higher affinity sFv.*

Significant sequence variability (six different amino acids) was observed at residues 93, and 94, with less variability (three different amino acids) at residues 95 and 95a. Thus a subset of the randomized residues appear to be more important in modulating affinity. All but one of these four residues (V<sub>L</sub>95) appear to have solvent accessible side chains in the C6.5 model. Three of the residues randomized (A89, W91, and G96) were 100% conserved in all mutants sequenced. Two additional residues (A90S and D92E) showed only a single conservative substitution. These conserved

residues appear to have a structural role in the variable domain, either in maintaining the main chain conformation of the loop, or in packing on the  $V_H$  domain. Residues A89, W91, and D92 are identical in both C6.5 and KOL, with conservative substitutions A90S and G96A observed at the other two positions in KOL, consistent with a structural role.

5 In the model of C6.5 indicated by this invention, G95b is in a turn and A89, A90, and W91 pack against the  $V_H$  domain at the  $V_H$ - $V_L$  interface. Hydrogen bonds between  $V_L$ D92 and  $V_L$ S27a and  $V_L$ N27b bridge L3 and L1 to stabilize the L3 and L1 conformations.

## 10 2) Mutation of C6ML3-9 sFv $V_H$ CDR3

### *Library construction and selection.*

To further increase the affinity of C6.5, we chose to mutate the  $V_H$  CDR3 of the highest affinity sFv (C6ML3-9,  $K_d = 1.0 \times 10^{-9}$  M) isolated from the C6VLCADR3 library, rather than mutate C6.5  $V_H$  CDR3 independently and combine mutants. This sequential approach was taken since the kinetic effects of independently isolated antibody fragment mutations are frequently not additive (Yang *et al.* (1995) *J. Mol. Biol.*, 254: 392-403).

Due to the length of the C6.5  $V_H$  CDR3 (20 amino acids), a high resolution functional scan was performed on C6.5 sFv in an attempt to reduce the number of amino acids subjected to mutation. Residues 95-99, 100a-100d, and 100g-102 were separately mutated to alanine, and the  $K_d$  of the mutated sFv determined. Residue 100f (alanine) was not studied. Residues 100 and 100e are a pair of cysteines separated by four amino acids. A homologous sequence in KOL (Marquardt *et al.* (1980) *supra.*) results in a disulfide bond between the two cysteines and a four residue miniloop. Therefore the two cysteines were simultaneously mutated to serine.

Results of the alanine scan are shown in Table 13. No detectable binding to c-erbB-2 ECD could be measured by BIAcore for C6.5H95A, C6.5W100hA, and C6.5E100jA. Three additional alanine mutants (G98A, Y100kA, and F100lA) yielded sFv with 20 fold to 100 fold higher  $K_d$  than wt sFv. Substitution of the two cysteines by alanine (100, 100e) yielded an sFv with an 17.5 fold higher  $K_d$ , and a much faster  $k_{off}$  ( $1.38 \times 10^{-1} s^{-1}$ ) than wt C6.5. The remainder of the alanine substitutions yielded only minor (0.5 to 3.7 fold) increases or decreases in  $K_d$ .

Based on the results of the alanine scan and a model of C6.5 based on the Fab KOL (Marquardt *et al.*, 1980), residues H95A, C100, and C100e were not mutated due to their probability of having an important structural role. H95 is likely to be buried at the  $V_H$ - $V_L$  interface where it makes critical packing contacts with the  $V_L$  domain. The two cysteine residues also are likely to have a structural role in maintaining the miniloop conformation. W100h was also not mutated given the unique features of tryptophan in antibody combining sites (Mian *et al.* (1991) *J. Mol. Biol.*, 217: 133-151).

The remaining 16 amino acids were completely randomized four residues at a time in four separate C6VHCDR3 libraries (96-99, library A; 100a-100d, library B; 100f, 100g, 100i, and 100j, library C, and 100k-102, library D; see Table 14). After transformation, libraries were obtained with sizes  $1.7 \times 10^7$  (library A),  $1.3 \times 10^7$  (library B),  $3.0 \times 10^6$  (library C), and  $2.4 \times 10^7$  (library D). The mutant phage antibody libraries were designated C6VHCDR3 libraries A, B, C, and D. PCR screening and DNA sequencing

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Table 13. Binding kinetics of C6.5 V<sub>H</sub> CDR3 mutants obtained by high resolution functional scan. Amino acid residues 95-99, 100a-100d, and 100g-102 of C6.5 V<sub>H</sub> CDR3 were mutated to alanine using site directed mutagenesis. Cysteine residues, C100 and C100e, were simultaneously mutated to serine.  $k_{on}$ , and  $k_{off}$  were measured by SPR in a BIAcore, and the  $K_d$  calculated. Numbering is according to Kabat *et al.* (1987). NB=no binding.

sFv clone	<u>Kd (mutant)</u> Kd (C6.5)	$K_d$ [10 <sup>-8</sup> M]	$k_{on}$ [10 <sup>5</sup> M <sup>-1</sup> s <sup>-1</sup> ]	$k_{off}$ [10 <sup>-2</sup> s <sup>-1</sup> ]
C6.5H95A	NB	NB	NB	NB
C6.5D96A	2.8	4.5	2.2 ± 0.34	1.0 ± 0.02
C6.5V97A	3.0	4.8	3.1 ± 0.62	1.5 ± 0.02
C6.5G98A	19.8	31.7	4.1 ± 0.71	13 ± 0.55
C6.5Y99A	3.7	5.9	9.0 ± 0.17	5.3 ± 0.07
C6.5C100S/C100eS	17.5	28.0	5.0 ± 0.25	13.8 ± 0.71
C6.5S100aA	1.8	2.8	4.7 ± 0.55	1.3 ± 0.04
C6.5S100bA	2.9	4.7	3.4 ± 0.49	1.6 ± 0.07
C6.5S100cA	1.5	2.4	4.5 ± 0.62	1.1 ± 0.03
C6.5N100dA	1.8	2.9	4.1 ± 0.34	1.2 ± 0.05
C6.5K100gA	0.6	0.98	4.3 ± 0.31	0.42 ± 0.01
C6.5W100hA	NB	NB	NB	NB
C6.5P100iA	0.6	1.0	10.5 ± 0.12	1.1 ± 0.02
C6.5E100jA	NB	NB	NB	NB
C6.5Y100kA	101.0	161.6	0.73 ± 0.07	11.8 ± 0.25
C6.5F100lA	28.4	45.4	1.1 ± 0.13	5.0 ± 0.06
C6.5Q101A	0.5	0.82	12.0 ± 0.02	0.98 ± 0.02
C6.5H102A	1.2	1.9	5.9 ± 0.57	1.1 ± 0.02

revealed that 100% of clones from all four libraries had full length insert and that the sequences were diverse (results not shown). Prior to selection, the percent of clones expressing sFv which bound c-erbB-2 ECD by ELISA was 1% for C6VHCDR3 library

A, 57%, library B, 2% library C, and 3% library D. The C6VHCDR3 libraries A, B, C, and D were selected on biotinylated c-erbB-2 ECD as described above, but using lower antigen concentration. The first round of selection was performed using  $5.0 \times 10^{-9}$  M c-erbB-2 ECD, tenfold lower than for the first round of selection of the C6VLCDR3 library. This concentration was chosen because the parental sFv for these libraries (C6ML3-9) had a greater than tenfold lower  $K_d$  than the parental clone for the C6VLCDR3 library (C6.5). Biotinylated c-erbB-2 ECD concentration was then decreased 100 fold for the second round of selection ( $5.0 \times 10^{-11}$  M) and tenfold for the third and fourth rounds ( $5.0 \times 10^{-12}$  M and  $5.0 \times 10^{-13}$  M). As for the C6VLCDR3 library, the rate of binding of polyclonal phage was measured in a BIAcore to determine the antigen concentration used for the subsequent round of selection as discussed below.

*Characterization of mutant sFv.*

After four rounds of selection, positive clones were identified by ELISA and at least 24 sFv from the fourth round of selection were ranked by  $k_{off}$  using SPR in a BIAcore. The ten sFv with the lowest  $k_{off}$  from C6VHCDR3 libraries A, C, and D were sequenced.

Table 14. Sequences, affinities and binding kinetics of sFv isolated from heavy chain CDR3 libraries A, B, C, and D.  $k_{on}$  and  $k_{off}$  were determined in a BIAcore using purified sFv, and  $K_d$  calculated. Dashes indicate sequence identity. Mutations arising from PCR error and located outside VH CDR3 are listed under the heading "other mutations". F=frequency of isolated sFv. \*  $k_{off}$  determined from unpurified sFv samples. Underline indicates mutated residue.

Clone Name	VH CDR3 sequence	Other Mutations	$k_d$ (M)	$k_{off}$ ( $s^{-1}$ )
C6.5	HDVGYC <del>SS</del> SNCAKWPEYFQH		$160.0 \times 10^{-10}$	$3.0 \times 10^{-5}$
VH CDR3 library A:				
C6ML3-9 (wt)	- <u>D</u> VGY-----			
C6ML3-A2	HDVGF <del>C</del> SSSNCAKWPEYFQH			
C6ML3-A3	HDVGYC <del>SS</del> S <del>D</del> CAKWPEYFQH		$160.0 \times 10^{-10}$	$3.0 \times 10^{-5}$
VH CDR3 library B:				
C6ML3-9 (wt)	----- <u>S</u> SSN-----		$10.0 \times 10^{-10}$	$7.6 \times 10^{-4}$
C6MH3-B1	HDVGYC <u>T</u> DRTCAKWPEYFQH		$1.6 \times 10^{-10}$	$6.7 \times 10^{-5}$

	C6MH3-B15	HDVGYC <u>CESSR</u> CAKWPEYFQH		7.7 X 10 <sup>-10</sup>	2.9 X 10 <sup>-4</sup>
	C6MH3-B11	HDVGYCSD <u>RS</u> CAKWPEYFQH		2.2 X 10 <sup>-10</sup>	2.3 X 10 <sup>-4</sup>
	C6MH3-B9	HDVGYC <u>KTAAC</u> AKWPEYFQH		8.7 X 10 <sup>-10</sup>	3.3 X 10 <sup>-4</sup>
	C6MH3-B8	HDVGYC* <u>TER</u> CAKWPEYFQH		7.2 X 10 <sup>-10</sup>	2.9 X 10 <sup>-4</sup>
5	C6MH3-B2	HDVGYC <u>TDP</u> RCAKWPEYFQH		3.1 X 10 <sup>-9</sup>	3.1 X 10 <sup>-4</sup>
	C6MH3-B39	HDVGYC <u>TDP</u> TCAKWPEYFQH		3.2 X 10 <sup>-10</sup>	1.9 X 10 <sup>-4</sup>
	C6MH3-B25	HDVGYC <u>LTT</u> RCAKWPEYFQH		3.6 X 10 <sup>-10</sup>	1.9 X 10 <sup>-4</sup>
	C6MH3-B21	HDVGYC <u>TTPL</u> CAKWPEYFQH		7.3 X 10 <sup>-10</sup>	2.4 X 10 <sup>-4</sup>
	C6MH3-B20	HDVGYC <u>SPAR</u> CAKWPEYFQH		8.7 X 10 <sup>-10</sup>	1.6 X 10 <sup>-4</sup>
10	C6MH3-B16	HDVGYC <u>ADV</u> RCAKWPEYFQH		3.1 X 10 <sup>-10</sup>	2.8 X 10 <sup>-4</sup>
	C6MH3-B47	HDVGYC <u>DRST</u> CAKWPEYFQH		1.1 X 10 <sup>-10</sup>	0.75 X 10 <sup>-4</sup>
	C6MH3-B48	HDVGYC <u>TDP</u> STCAKWPEYFQH		2.3 X 10 <sup>-10</sup>	1.3 X 10 <sup>-4</sup>
	C6MH3-B5	HDVGYC <u>TDAT</u> TCAKWPEYFQH		3.4 X 10 <sup>-10</sup>	2.3 X 10 <sup>-4</sup>
	C6MH3-B41	HDVGYC <u>DRPT</u> CAKWPEYFQH		5.3 X 10 <sup>-10</sup>	2.7 X 10 <sup>-4</sup>
15	C6MH3-B2	HDVGYC <u>TDP</u> RTCAKWPEYFQH		5.8 X 10 <sup>-10</sup>	3.2 X 10 <sup>-4</sup>
	C6MH3-B27	HDVGYC <u>KNS</u> RTCAKWPEYFQH		4.7 X 10 <sup>-10</sup>	4.0 X 10 <sup>-4</sup>
	C6MH3-B34	HDVGYC <u>QDTR</u> TCAKWPEYFQH	VL Q1R	ND	ND
	C6MH3-B43	HDVGYC <u>EDYT</u> TCAKWPEYFQH		ND	ND
	C6MH3-B46	HDVGYC <u>TPRT</u> CAKWPEYFQH	VH K23Q VH V76G	ND	ND
20	C6MH3-B33	HDVGYC <u>SDQT</u> TCAKWPEYFQH		ND	ND
	C6MH3-B31	HDVGYC <u>DDYT</u> TCAKWPEYFQH	VL P7L	ND	ND
	VH CDR3 library C:				
25	C6ML3-9 (wt)	-----AKWPE----		10.0 X 10 <sup>-10</sup>	7.6 X 10 <sup>-4</sup>
	C6MH3-C4	HDVGYC <u>SSS</u> NC <u>AV</u> WPEYFQH		3.7 X 10 <sup>-10</sup>	2.0 X 10 <sup>-4</sup>
	C6MH3-C3	HDVGYC <u>SSS</u> NC <u>AK</u> WPEYFQH	VH G15E VL N54D	6.5 X 10 <sup>-10</sup>	3.2 X 10 <sup>-4</sup>
	VH CDR3 library D:				
30	C6ML3-9 (wt)	HDVGYC <u>SSS</u> NC <u>AK</u> WPEYFQH		10.0 X 10 <sup>-10</sup>	7.6 X 10 <sup>-4</sup>
	C6MH3-D2	HDVGYC <u>SSS</u> NC <u>AK</u> WPE <u>W</u> LGV		1.6 X 10 <sup>-10</sup>	2.0 X 10 <sup>-4</sup>
	C6MH3-D3	HDVGYC <u>SSS</u> NC <u>AK</u> WPE <u>W</u> LDN		2.7 X 10 <sup>-10</sup>	2.5 X 10 <sup>-4</sup>
	C6MH3-D6	HDVGYC <u>SSS</u> NC <u>AK</u> WPE <u>W</u> MYP		3.5 X 10 <sup>-10</sup>	1.8 X 10 <sup>-4</sup>
35	C6MH3-D5	HDVGYC <u>SSS</u> NC <u>AK</u> WPE <u>W</u> MQM		3.8 X 10 <sup>-10</sup>	2.1 X 10 <sup>-4</sup>

C6MH3-D1	HDVGYCSSSNCAKWPEWLHV		$3.1 \times 10^{-10}$	$1.1 \times 10^{-4}$
C6MH3-D7	HDVGYCSSSNCAKWPEWQDP		ND	$3.1 \times 10^{-4}$

5 Due to the diversity of isolated sFv in C6VHCDR3 library B, 48 sFv were ranked by  $k_{\text{off}}$  using SPR, and 22 clones with the lowest  $k_{\text{off}}$  were sequenced. Single-chain Fv were purified by IMAC, followed by gel filtration to remove any dimeric or aggregated sFv. The  $k_{\text{on}}$  and  $k_{\text{off}}$  were determined by BIAcore and the  $K_d$  calculated.

10 Very different results were obtained from the four libraries with respect to the number of higher affinity sFv isolated, and the value of the highest affinity sFv. The best results were obtained from library B (Table 14). Fifteen sFv were isolated with a  $K_d$  lower than wt C6ML3-9 and no wt sequences were observed (Table 14). The best sFv (C6MH3-B47) had a  $K_d = 1.1 \times 10^{-10}$  M, ninefold lower than C6ML3-9 and 145 fold lower than C6.5. The  $k_{\text{off}}$  of this sFv was  $7.5 \times 10^{-5} \text{ s}^{-1}$ , tenfold lower than C6ML3-9 and 84 fold lower than C6.5. While a wide range of sequences was observed (Table 14, library B), a subset of sFv had the consensus sequence TDRT (first eight sFv, Table 14). The consensus sequence is identical with the sequence of C6MH3-B1, which is the sFv with the lowest  $k_{\text{off}}$  ( $6.0 \times 10^{-5} \text{ s}^{-1}$ ).

15 Five sFv were isolated that had a  $k_{\text{off}}$  2.5 to 3.75 fold lower than C6ML3-9, however expression levels were too low to obtain adequate purified sFv for measurement of the  $K_d$  (last five sequences, Table 14, library B). The next best results were obtained from library D (Table 14). Five higher affinity sFv were isolated, with the best having a  $K_d$  sevenfold higher than wt C6ML3-9. An additional sFv was isolated that had a  $k_{\text{off}}$  lower than wt sFv, however the expression level was too low to obtain adequate purified sFv for measurement of the  $K_d$  (last sequence, Table 14, library D).

25 There was selection for a consensus mutation of Y100kW and replacement of F100I with hydrophobic methionine or leucine. No higher affinity sFv were isolated from either the A or C libraries. From library A, 8/10 sFv were wild-type, with one higher affinity sFv, a contaminant from library B. A single mutant sFv with the conservative replacement of Y99F had an apparent  $k_{\text{off}}$  2.5 times lower than wt, but expression levels were too low to obtain adequate purified sFv to measure the  $K_d$ . From library C, 8/10 sFv were wt sFv, with one higher affinity sFv having mutations located in the  $V_H$  and  $V_L$  genes, but not in the region intentionally mutated. The isolated mutant sFv K100gV had

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a  $K_d$  2.7 fold lower than wt ( $k_{off}$  3.8 fold lower than C6ML3-9), correlating with the data of the alanine scan, in which K100gA was the only sFv with decreased  $k_{off}$ .

*Ability of alanine scanning to identify residues which modulated affinity.*

5 Residue E100j, the only residue that when converted to alanine had no detectable binding, was 100% conserved. Otherwise, there was no correlation between the frequency with which the wt amino acid was recovered and the extent to which binding was reduced by substitution to alanine. Similarly, there was no correlation between residues shown to modulate affinity by alanine scanning and mutations exhibiting

10 improved binding. This is clear when comparing the results obtained from library B (where no alanine mutant had more than a 2.9 fold increase in  $K_d$ ) and library D (where  $K_d$  was markedly increased for two alanine mutants, Y100kA and F100IA). Despite the different alanine scan results, both libraries yielded similar nine and sevenfold increases in affinity. This result appears to be different than the results of Lowman *et al.* (1993)

15 *J. Mol. Biol.*, 234: 564-578, who found a mild ( $R^2=0.71$ ) positive correlation between the frequency with which the wt amino acid was recovered from a phage library of human growth hormone mutants and the extent to which binding was reduced by alanine scanning. In addition, their largest improvements in affinity were for those residues shown by alanine scanning to significantly affect binding.

20 The reason for the different results is unclear, however in two of the  $V_H$  CDR3 libraries where alanine scanning indicated a significant effect on binding (libraries A and C), expression levels of mutants were generally low. This could have affected the selection results.

25 **3) Correlation between affinity and cell surface retention of sFv.**

The retention of biotinylated C6.5, C6ML3-9, and C6MH3-B1 sFv on the surface of SK-OV-3 cells expressing c-erbB-2 was determined, both to verify the observed differences in  $k_{off}$ , and to confirm that the antigen as presented in the BIAcore had biologic significance. The half life ( $t_{1/2}$ ) of the sFv on the cell surface was much less

30 than 5 min for C6.5, 11 min for C6ML3-9, and 102 min for C6MH3-B1. These values agree closely with the  $t_{1/2}$  calculated from the  $k_{off}$  as determined by SPR in a BIAcore (1.6 min for C6.5, 13 min for C6ML3-9, and 135 min for C6MH3-B1). The anti-digoxin sFv 26-10 (Huston *et al.* (1988) *supra.*) was used as negative control, and

no binding to c-erbB-2 ECD in a BIAcore or to c-erbB-2 on SK-OV-3 cells was observed.

#### Example 4

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#### Elution of Antibodies

As higher affinity phage antibodies are generated, it becomes more difficult to elute them from c-erbB-2. Selection of the highest affinity mutants is enhanced when elution conditions are optimized. To determine optimal elution conditions, the C6.5 V1 CDR3 mutant library was selected on c-erbB-2, and a number of different elution conditions studied (infecting directly off of magnetic beads, 10 mM HCl, 50 mM HCl, 100mM HCl, 2.6 M MgCl<sub>2</sub>, 4 M MgCl<sub>2</sub>, 100 mM TDA, and with 1 μM c-erbB-2). The greatest percentage of clones with a  $k_{off}$  slower than C6.5 was obtained when eluting with 50 mM HCl, 100 mM HCl, or 4 M MgCl<sub>2</sub>. Even after the eluted clones were screened by BIAcore to identify those with the slowest  $k_{off}$ , the highest affinity clones resulted from elutions performed with 100 mM HCl as shown in Table 15 (in this experiment 4 mM MgCl<sub>2</sub> was not examined).

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These results correlated with the amount of phage antibody library that remained bound in the BIAcore after using one of the different elution conditions. For the V<sub>H</sub> CDR3 elutions phage were eluted sequentially with 4 mM MgCl<sub>2</sub> and 100 mM HCl. As affinity increases further more stringent elution conditions may be required. This can be determined by analyzing phage libraries in the BIAcore.

**Table 15.** Results of C6.5 L3 randomization 4th round off-rate selection and elution. Underlines indicate mutated amino acids.

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Clones	F	CDR3 Sequence	$k_d$ (M)	$k_{off}$ (s <sup>-1</sup> )
C6.5		AAWDDSLSGWV	$1.6 \times 10^{-8}$	$6.3 \times 10^{-3}$
Elution with 100 mM HCl:				
C6ML3-5	4	AAWD <u>Y</u> SLSGWV	$3.7 \times 10^{-9}$	$6.3 \times 10^{-3}$
C6ML3-9		A <u>S</u> WDY <u>T</u> LSGWV	$1.0 \times 10^{-9}$	$1.9 \times 10^{-4}$
C6ML3-14	2	AAWDD <u>P</u> L <u>W</u> GWV	$1.1 \times 10^{-9}$	$7.6 \times 10^{-4}$
C6ML3-15		AAWDR <u>P</u> L <u>W</u> GWV	$2.2 \times 10^{-9}$	$7.7 \times 10^{-3}$
Elution with 2.6 M MgCl <sub>2</sub> :				
C6ML3-5	2	AAWD <u>Y</u> SLSGWV	$3.7 \times 10^{-9}$	$1.9 \times 10^{-3}$
C6ML3-7	2	AAWD <u>Y</u> AVSGWV	$2.6 \times 10^{-9}$	$1.7 \times 10^{-3}$

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		AAWDYSRSGWV	$1.6 \times 10^{-9}$	$7.2 \times 10^{-4}$
	2	ASWDYYRSGWV	$5.0 \times 10^{-9}$	$1.7 \times 10^{-3}$
		AAWDRPLWGWV	$2.2 \times 10^{-9}$	$1.3 \times 10^{-3}$
5	Elution with 100 mM tirethylamine:			
	3	AAWDYSLSGWV	$3.7 \times 10^{-9}$	$1.9 \times 10^{-3}$
	2	AAWDYSRSGWV	$1.6 \times 10^{-9}$	$7.2 \times 10^{-4}$
		ASWDASLWGWV	$2.4 \times 10^{-9}$	$6.2 \times 10^{-4}$
		ASWDRPLWGWV	$1.5 \times 10^{-9}$	$1.0 \times 10^{-3}$
10		AAWEQSLWGWV	$3.0 \times 10^{-9}$	$1.4 \times 10^{-3}$
	Elution with 10 mM HCl:			
		AAWDYSLSGWV	$3.7 \times 10^{-9}$	$1.9 \times 10^{-3}$
		AAWDYAVSGWV	$2.6 \times 10^{-9}$	$1.7 \times 10^{-3}$
15		AAWDYSQSGWV	$4.5 \times 10^{-9}$	$2.2 \times 10^{-3}$
		AAWDASLSGWV	$8.3 \times 10^{-9}$	$3.6 \times 10^{-3}$
		ASWDHSLWGWV	$1.5 \times 10^{-9}$	$1.0 \times 10^{-3}$
		AAWDEQIFGWV	$12.4 \times 10^{-9}$	$7.9 \times 10^{-3}$
		AAWDNRHSGWV	$7.4 \times 10^{-9}$	$4.4 \times 10^{-3}$
20		AAWDDSRSGWV	$8.3 \times 10^{-9}$	$5.0 \times 10^{-3}$
	Elution with 50 mM HCl:			
		ASWDYSLSGWV	$3.2 \times 10^{-9}$	$1.9 \times 10^{-3}$
		AAWDYAVSGWV	$2.6 \times 10^{-9}$	$1.7 \times 10^{-3}$
25		AAWDYSRSGWV	$1.6 \times 10^{-9}$	$7.2 \times 10^{-4}$
		ASWDYYRSGWV	$5.0 \times 10^{-9}$	$1.7 \times 10^{-3}$
		TAWDYSLSGWV	no expression	
		ASWDYALSGWV	$2.5 \times 10^{-9}$	$1.7 \times 10^{-3}$
		AAWDGTLWGWV	$1.7 \times 10^{-9}$	$2.2 \times 10^{-3}$
30	Elution with 1 $\mu$ M c-erbB-2 ECD for 30 minutes			
	5	AAWDYSLSGWV	$3.7 \times 10^{-9}$	$1.9 \times 10^{-3}$
		AAWDYALSGWV	no expression	
35	3	ASWDYYLIGWV	no expression	

For example, in a second experiment, polyclonal phage were prepared after three rounds of selection of the C6VLCDR3 library and studied using SPR in a BIAcore. After an initial bulk refractive index change, binding of phage to immobilized c-erbB-2 ECD was observed, resulting in an average of 189 RU bound. Phage were

then allowed to either spontaneously dissociate from c-erbB-2 ECD using hepes buffered saline (HBS) as running buffer, or were eluted with either 100 mM HCl, 50 mM HCl, 10 mM HCl, 2.6 M MgCl<sub>2</sub>, or 100 mM TEA.

5 Major differences were observed between eluents in their ability to remove bound phage. The most effective solutions in removing bound phage antibodies were 100 mM HCl and 50 mM HCl, followed by 100 mM TEA. 2.6 M MgCl<sub>2</sub> (which removes 100% of wild type C6.5) and 10 mM HCl were only minimally more effective than the running buffer in removing bound phage.

10 These results demonstrate the important effect of eluent choice on the affinities of selected antibodies, even when using limiting antigen concentration and BIAcore screening to identify the highest affinity sFv. Two previously described elution regimens were found to be the least effective for selecting higher affinity antibodies; infecting without elution by adding magnetic beads with antigen-bound phage directly to *E. coli* cultures (Figini *et al.* (1994) *J. Mol. Biol.*, 239: 68) and competitive elution of sFv with soluble antigen (Hawkins *et al.* (1992) *J. Mol. Biol.*, 226: 889; Clackson *et al.* (1991) *Nature*, 352: 624; Riechmann *et al.* (1993) *Biochemistry*, 32: 8848).

15 When eluting by incubating phage bound to antigen with *E. coli*, it is believed the phage must dissociate from antigen for infection to occur. Steric hindrance, due to the size of paramagnetic beads, blocks the attachment of pIII on antigen bound phage to the f-pilus on *E. coli*. This would result in preferential selection of sFv with rapid  $k_{off}$ , consistent with the present results. Since a reduction in  $k_{off}$  is the major mechanism for decreases in  $K_d$ , this results in the selection of lower affinity sFv.

20 Eluting with soluble antigen has a similar effect on the kinetics of selected sFv. The phage must first dissociate from immobilized antigen, then rebinding is blocked by binding of the phage to soluble antigen. Phage antibodies with the lowest  $k_{off}$  will remain bound to immobilized antigen and therefore are not available for infection of *E. coli*.

25 The optimal type of eluent (acidic, basic, chaotropic) and concentration required will depend on the phage antibody affinity (Lewis *et al.* (1985) *J. Steroid. Biochem.* 22: 387; Parini *et al.* (1995) *Analyst*, 120: 1153) and the type of bonds that need to be interrupted. This will vary considerably between libraries, depending on the nature of the antigen-antibody interaction.

In this example, significantly higher affinity sFv were obtained eluting with HCl, pH 1.3 compared to HCl, pH 2.0. In fact, the affinities of sFv isolated after elution with HCl, pH 2.0 were no different than results obtained without eluting. Similarly, 2.6 M MgCl<sub>2</sub> was studied because it was previously determined (see above) that it would remove 100% of bound wild type C6.5. This concentration of MgCl<sub>2</sub>, however, was ineffective in eluting C6.5 V<sub>L</sub> CDR3 mutants. Eluting with higher concentrations of MgCl<sub>2</sub> would have resulted in the selection of higher affinity sFv. For example, 3 M MgCl<sub>2</sub> was required to elute 100% of C6L1 sFv ( $K_d = 2.5 \times 10^{-9}$  M) from a c-erbB-2 ECD BIAcore sensor chip and 4 M MgCl<sub>2</sub> was required to elute 100% of C6ML3-9 ( $K_d = 1.0 \times 10^{-9}$  M).

A convenient way to predict the optimal eluent is to analyze polyclonal phage in a BIAcore. The results can then be used to design elution conditions to achieve optimal enrichment for high affinity clones. One approach is to elute sequentially, using a less stringent eluent to remove low affinity binders, followed by a more stringent eluent to remove high affinity binders. Thus the BIAcore information is used to select 'washing' reagents which remove low affinity phage antibodies more effectively than PBS. This will reduce the number of selection rounds and amount of screening required to select and identify the highest affinity binders.

This strategy is also be useful to isolate antibodies to low density antigens on intact cells or tissue. A mild eluent could be used to remove low affinity phage antibodies, which are preferentially selected due to high density antigen present on the cell surface, as well as non-specifically bound phage. Phage specific for lower density antigens would then be removed using a more stringent solution.

An alternative to eluting with stringent solutions is to use antigen biotinylated with NHS-SS-Biotin (Pierce) (Griffiths *et al.* (1994) *EMBO J.*, 13: 3245). All of the bound phage can be released from the magnetic beads by reducing the disulfide bond between antigen and biotin. One advantage of this approach is that elution of all phage is guaranteed. Use of NHS-SS-Biotin could be combined with use of a milder eluent for washing (determined by BIAcore analysis) to increase enrichment for higher affinity phage antibodies.

The present experiments suggest, however, that use of stringent eluents that are chemically different (acidic, basic, or chaotropic) results in the selection of sFv of equally high affinity, but of different sequence. Isolation of sFv of different

sequences has a number of advantages. Single amino acid changes can affect expression levels in *E. coli* dramatically. For example, expression level of C6ML3-5 (100  $\mu\text{g/L}$ ) was 100 times less than for wild type C6.5 (10 mg/L). Furthermore, different sFv might have different physicochemical characteristics (dimerization, stability, or immunoreactivity) or even different effects *in vivo* (specificity, biodistribution, or clearance). Thus parallel selections using different stringent eluents should result in a greater number of high affinity binders than use of a single eluent.

### Example 5

#### 10      Production of Antibodies Combining C6MH3-B1 or C6MH3-B47 with D Library (YFOH) Mutations

##### I. Methods.

##### *Construction of sFv combining higher affinity $V_H$ and $V_L$ genes.*

The  $V_L$  CDR3 gene sequences of the two highest affinity sFv isolated from the C6VLCDR3 library (C6ML3-9 or C6ML3-12) were combined with the highest affinity sFv previously obtained from light chain shuffling (C6L1,  $K_d = 2.5 \times 10^{-9}$  M). The C6L1 plasmid (10 ng/ $\mu\text{l}$ ) was used as a template for PCR amplification using primers LMB3 and either PML3-9 or PML3-12 (Table 18). The gel purified PCR fragments were reamplified using primers LMB3 and *HuJl* 2-3ForNot (Marks *et al.* (1991) *supra.*) to introduce a *NotI* restriction site at the 3'-end of the sFv. The gel purified PCR fragments were digested with *NcoI* and *NotI* and ligated into pUC119 *Sfi*-*NotmycHis* digested with *NcoI* and *NotI*. The resulting sFv were designated C6-9L1 and C6-12L1. The  $V_L$  genes of C6-9L1 and C6-12L1 were combined with the  $V_H$  genes of the two highest affinity sFv from the C6VHCDR3 libraries (C6MH3-B1 and C6MH3-B47). The rearranged  $V_H$  genes of C6MH3-B1 and -B47 were amplified by PCR using the primer LMB3 and PC6VH1FOR, digested with *NcoI* and *XhoI* (located in FR4 of the heavy chain) and ligated into C6-9L1 or C6-12L1 digested with *NcoI* and *XhoI* to create C6-B1L1 and C6-B47L1. The heavy chain of C6MH3-B1 or C6MH3-B47 was amplified by PCR using LMB3 and one of the PCD primer (PCD1, PCD2, PCD3, PCD5, or PCD6; Table 18) to construct combinations of sFv from the C6VHCDR3B and D libraries. The purified PCR fragments were spliced with the  $V_L$  fragment of C6ML3-9 (VHD2) that was used to create the C6VHCDR3D library exactly as described above. The full length sFv gene was digested with *NcoI* and *NotI* and

ligated into pUC119 Sfi-NotmycHis. Clones were termed C6-B1D1, -B1D2, -B1D3, -B1D5, -B1D6, -B47D1, -B47D2, -B47D3, -B47D5, and -B47D6. Colonies were screened for the presence of the correct insert by PCR fingerprinting and confirmed by DNA sequencing. Single-chain Fv were expressed, purified, and affinities determined by SPR, as described above.

## II. Results.

### *Effects on binding kinetics by combining mutations from high affinity sFv.*

As described above, to further increase affinity, the sequences of the two highest affinity sFv obtained from the VH CDR3B library (C6MH3-B1 or C6MH3-B47) were combined with the sequences of sFv isolated from the C6VHCDR3D library (C6MH3-D1, -D2, -D3, -D5, or -D6). An increase in affinity from wild-type was obtained for all these combinations, yielding an sFv (C6-B1D3) that had a 1230 fold lower  $K_d$  than wt C6.5 (Table 16). The extent of additivity varied considerably, however, and could not be predicted from the parental  $k_{on}$ ,  $k_{off}$ , or  $K_d$ . In some combinations, cooperativity was observed, with a negative  $\Delta\Delta G_f$ . Additional combinations were made between a previously described light chain shuffled C6.5 mutant (C6L1, sixfold decreased  $K_d$ ) and one of two  $V_L$  CDR3 mutants (C6ML3-9 and C6ML3-12). These combinations yielded sFv with 49 and 84 fold improved affinity (Table 16). Introducing the same rearranged  $V_L$  gene into the highest affinity  $V_H$  CDR3 mutants (C6MH3-B1 or C6MH3-B47) resulted in decreased affinity compared to C6MH3-B1 (Table 5).

Table 16. Binding kinetics of sFv derived from C6.5  $V_L$  CDR3 and  $V_H$  CDR3 mutants. Mutants obtained by combining mutations of C6MH3-B1 or C6MH3-B47 with mutations from D library clones (D1, D2, D3, D5, D6). Rate constants  $k_{on}$ , and  $k_{off}$  were measured by SPR in a BIAcore, and the  $K_d$  calculated.

Clone	$K_d$ [ $10^{-10}$ M]	$k_{on}$ [ $10^3$ s <sup>-1</sup> M <sup>-1</sup> ]	$k_{off}$ [ $10^{-4}$ s <sup>-1</sup> ]	$\frac{K_d}{K_d \text{ (parent)}}$ $\frac{K_d \text{ (parent)}}{K_d \text{ (mut)}}$	$\frac{K_d \text{ (C6.5)}}{K_d \text{ (mut)}}$	$\Delta\Delta G_f$ [kcal/mol]
A. Combined mutants: C6ML3-9 OR C6ML3-12 with light chain shuffled C6L1:						
C6-9L1	3.3	9.2 ± 0.20	3.0 ± 0.40	3.0	49	+ 0.42
C6-12L1	1.9	6.7 ± 0.12	1.3 ± 0.32	8.4	84	- 0.18
B. Combined mutants: C6MH3-B1 OR C6MH3-B47 with light chain shuffled C6L1:						
C6-B1L1	6.3	3.8 ± 0.19	2.4 ± 0.01	0.19	25	+ 0.43
C6-B47L1	6.0	3.0 ± 0.16	1.8 ± 0.01	0.18	27	+ 0.45

C. Combined mutants: C6MH3-B1 OR C6MH3-B47 with D library mutants:						
C6-B1D1	0.32	4.7 ± 0.31	0.15 ± 0.005	3.8	500	- 0.61
C6-B1D2	0.15	6.9 ± 0.42	0.10 ± 0.014	8.0	1067	- 0.07
C6-B1D3	0.13	6.4 ± 0.20	0.08 ± 0.002	9.2	1231	- 0.53
C6-B1D5	0.35	5.1 ± 0.36	0.18 ± 0.001	3.4	457	- 0.40
C6-B1D6	0.32	4.1 ± 0.17	0.13 ± 0.002	3.8	500	- 0.16
C6-B47D1	0.68	7.1 ± 0.95	0.48 ± 0.001	1.6	235	- 0.11
C6-B47D2	0.44	9.8 ± 0.72	0.43 ± 0.001	2.5	364	+ 0.62
C6-B47D3	0.48	6.6 ± 0.26	0.32 ± 0.001	2.3	333	+ 0.29
C6-B47D5	0.63	6.2 ± 0.31	0.39 ± 0.002	1.7	254	- 0.01
C6-B47D6	0.51	5.9 ± 0.30	0.30 ± 0.001	2.2	314	+ 0.17

### Example 6

#### Production of C6.5-Based Diabodies.

To improve tumor retention sFv dimers (sFv')<sub>2</sub> were created as described above by introducing a free cysteine at the C-terminus of the sFv. The dimer had a 40 fold improved affinity compared to the monomer ( $K_d = 4.0 \times 10^{-10}$  M). However, evaluation of the C6.5 (sFv')<sub>2</sub> *in vivo*, showed no significantly improved tumor retention at 24 hours. Without being bound to a theory, it is believed that the disulfide bond is being reduced *in vivo*, yielding monomeric sFv.

To obtain a stable molecule for evaluation *in vivo*, a C6.5 diabody (also a (sFv)<sub>2</sub>) was produced without introducing a cysteine and crosslinking. Instead, the diabody was produced as described in Holliger *et al.*, *Proc. Natl. Acad. Sci. USA.*, 90: 6444-6448 (1993) (*see also* WO 94/13804). To produce the C6.5 diabody, the peptide linker sequence between the V<sub>H</sub> and V<sub>L</sub> domains was shortened from 15 amino acids to 5 amino acids. This was done at the genetic level. Synthetic oligonucleotides encoding the 5 amino acid linker (G<sub>4</sub>S) were used to PCR amplify the C6.5 V<sub>H</sub> and V<sub>L</sub> genes, which were then spliced together to create the C6.5 diabody gene. The diabody gene was cloned into pUC119mycHis, the diabody expressed, and purified by IMAC followed by gel filtration as described above.

The affinity of the diabody was measured using surface plasmon resonance in a BIAcore and found to be  $4.2 \times 10^{-10}$  M, with a  $k_{off}$  of  $3.2 \times 10^{-4}$  s<sup>-1</sup>. The retention of the FITC labeled diabody on the surface of c-erbB-2 expressing cells was determined by FACS. After 180 minutes, 77% was still retained on the cell surface. Assuming an

exponential decay for binding, this value for cell surface retention correlates with a  $k_{off}$  of  $7 \times 10^{-5} \text{ s}^{-1}$ . This is significantly slower than the  $k_{off}$  measured on the BIAcore, and suggests that c-erbB-2 density is higher on the cell surface than the density used for the BIAcore measurements.

5

The retention of the C6.5 diabody in scid mice bearing subcutaneous SK-OV-3 tumors was compared to C6.5. Single chain Fv were radio-iodinated using the chloramine-T method, and 25  $\mu\text{g}$  injected into mice. Values are shown in Table 17 and plotted in Figure 4. At 24 hours, tumor retention was 6.48% of the injected dose/gm of tumor, compared to 0.98% for C6.5. Tumor:blood ratios were 9.7:1 for the diabody and 19.6:1 for the C6.5 sFv. Significant amounts (1.41%) of the diabody was retained at 72 hours. The total area under the curve (AUC) for tumor:blood was 2.3:1.

10

The ability of the C6.5 diabody to be internalized into c-erbB-2 expressing cells was compared to C6.5 sFv and higher affinity C6.5 mutants. Only the diabody was internalized, consistent with studies using monoclonal antibodies to c-erbB-2 which show that crosslinking of c-erbB-2 results in internalization. This does not occur with all anti-c-erbB-2 antibodies, but rather is epitope dependent. Thus C6.5 recognizes an internalizing epitope, but internalization only results when the receptor is crosslinked by the diabody. This opens up the possibility of creating diabody-toxin fusions (since toxins must be internalized to be active). It is believed that C6.5 also causes signalling through c-erbB-2 via cross-linking of the receptor and activation of the tyrosine kinase activity. It has been shown that activation of the cell through c-erbB-2 signalling increases the sensitivity of the cell to conventional cancer chemotherapeutics. Through activation of the kinase, C6.5 is expected to have therapeutic properties when combined with a conventional cancer chemotherapeutic.

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20

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Table 17. Tissue distribution of diabody as a function of time.

Time (Hrs)	C6.5			
	Tumor mean $\pm$ se	Blood mean $\pm$ se	Tumor mean $\pm$ se	Blood mean $\pm$ se
0.08		42.08 $\pm$ 0.77		
1	6.93 $\pm$ 0.39	21.47 $\pm$ 1.67		
4	10.06 $\pm$ 0.63	6.73 $\pm$ 0.29	0.98 $\pm$ 0.08	0.05 $\pm$ 0.01
24	6.48 $\pm$ 0.77	0.67 $\pm$ 0.05		

30

48	2.42 ± 0.18	0.11 ± 0.01		
72	1.41 ± 0.13	0.06 ± 0		

5 **Table 18.** Sequences of primers used in the foregoing examples. Nucleotide mixtures used, molar fraction: 1: A (0.7), C, G, and T (0.1); 2: C (0.7), A, G, and T (0.1); 3: G (0.7); 4: T (0.7), A, C, and G (0.1); 5: C and G (0.5); 6: C (0.7) and G (0.3); 7: C (0.3) and G (0.7); 8: A, C, G, and T (0.25).

Primer	Sequence
LMB3	5'-CAGGAAACAGCTATGAC-3'
fd-seq1	5'-GAATTTCTGTATGAGG-3'
PHEN=1seq	5'-CTATGCGGCCCATTC-3'
Linkseq	5'-CGATCCGCCACCGCCAG-3'
PVH1For1	5'-TCGCGCGCAGTAATACACGGCCGTGTC-3'
PVH3For1	5'-TCGCGCGCAGTAATACACAGCCGTGTCCTC-3'
PVH5For1	5'-TCGCGCGCAGTAATACATGGCGGTGTCGA-3'
PVH1For2	5'-GAGTCATTCTCGACTTGGCGCCGCTCGCGCGCAGTAATACACGGCCGTGTC-3'
PVH3For2	5'-GAGTCATTCTCGACTTGGCGCCGCTCGCGCGCAGTAATACACAGCCGTGTCCTC-3'
PVH5For2	5'-GAGTCATTCTCGACTTGGCGCCGCTCGCGCGCAGTAATACATGGCGGTGTCGA-3'
PC6VL1back	5'-AGCGCCGTGATTTTTGCGCGCACATGACGTGGGATATTGC-3'
RJH1/2/6Xho	5'-ACCCTGGTCACCGTCTCGAGTGGTGA-3'
RJH3Xho	5'-ACAATGGTCACCGTCTCGAGTGGTGA-3'
RJH4/5Xho	5'-ACCCTGGTCACCGTCTCGAGTGGTGA-3'
PC6VH1For	5'-GAGTCATTCTCGTCTCGAGACGGTGACCAGGGTGCC-3'
VL1	5'-GTCCCTCCGCCGAACACCCA,5,2,2,5,3,1,6,1,3,5,3,1,7,4,2,7,4,2,2,2,1,5,3,2,5,3,2,ACAGTAAT AATCAGCCTCAT-3'
VL2	5'-GAGTCATTCTCGACTTGGCGCCGACCTAGGACGGTCAGCTTGGTCCCTCCGCCGAACACCCA-3'
VHA	5'-GCGCAGTTGGAACACTGCA,5,8,8,5,8,8,5,8,8,5,8,8,ATGTCTCGCACAAAATACAGGC-3'
RVHA	5'-TGCAGTAGTCCAACCTGCGC-3'
VHB	5'-GTATTCAGGCCACTTTGCGCA,5,8,8,5,8,8,5,8,8,5,8,8,GCAATATCCCAGTCATGTC-3'
RVHB	5'-TGGCAAAGTGGCCTGAATAC-3'
VHC	5'-CTGGCCCAATGCTGGAAGTA,5,8,8,5,8,8,CCA,5,8,8,5,8,8,GCAAGTTGGAACACTGCAATATCC-3'
RVHC	5'-TACTTCCAGCATTGGGGCCAG-3'
VHD	5'-GACCGGGTGGCCCTGGCCCA,5,8,8,5,8,8,5,8,8,5,8,8,TTGAGGCCACTTTGCGCAGTTGG-3'
RVHD	5'-TGGGGCCAGGGCACCCCTGGTC-3'
C6hisnot	5'-GATACGGCACCGCGCACCTGGCGCCGATGGTGATGATGGTGATGTGCGGCACCTAGGACGGTCAGCTTGG-3'
PML3-9	5'-CCTAGGACGGTCAGCTTGGTCCCTCCGCCGAACACCAACCACTCAGGGTGAATCCCAGGATGCACAGTAATAATCAGC-3'
PML3-12	5'-CCTAGGACGGTCAGCTTGGTCCCTCCGCCGAACACCAACCACTCAGGGTGAATCCCAGGATGCACAG-3'
PCD1	5'-GACGGTGACCAGGGTGCCCTGGCCCAACGTCAGCCATTGAGCCACTTTGCGCA-3'
PCD2	5'-GACGGTGACCAGGGTGCCCTGGCCCAACGTCAGCCATTGAGCCACTTTGCGCA-3'
PCD3	5'-GACGGTGACCAGGGTGCCCTGGCCCAAGTTGTCCAACATTGAGCCACTTTGCGCA-3'
PCD5	5'-GACGGTGACCAGGGTGCCCTGGCCCAACATCTGCATCCATTGAGCCACTTTGCGCA-3'
PCD6	5'-GACGGTGACCAGGGTGCCCTGGCCCAAGGGTACATCCATTGAGCCACTTTGCGCA-3'

45 It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference.

**WHAT IS CLAIMED IS:**

- 1                   1.     A human antibody that specifically binds to c-erbB-2, said antibody  
2     being a C6 antibody.
- 1                   2.     The antibody of claim 1, wherein said antibody has the variable  
2     heavy ( $V_H$ ) chain of C6.5.
- 1                   3.     The antibody of claim 1, wherein said antibody has the variable  
2     light ( $V_L$ ) chain of C6.5.
- 1                   4.     The antibody of claim 1, wherein said antibody is C6.5.
- 1                   5.     The antibody of claim 1, wherein said antibody has the amino acid  
2     sequence of C6.5.
- 1                   6.     The antibody of claim 1, wherein said antibody has the amino acid  
2     sequence of C6ML3-14.
- 1                   7.     The antibody of claim 1, wherein said antibody has the amino acid  
2     sequence of C6L1.
- 1                   8.     The antibody of claim 1, wherein said antibody has the amino acid  
2     sequence of C6MH3-B1.
- 1                   9.     The antibody of claim 1, wherein said antibody has the amino acid  
2     sequence of C6ML3-9.
- 1                   10.    The antibody of claim 1, wherein said antibody is selected from the  
2     group consisting of an antibody having a  $V_L$  domain with one of the amino acid  
3     sequences shown in Table 10, an antibody having a  $V_H$  domain with one of the amino  
4     acid sequences shown in Table 12, an antibody having a  $V_L$  CDR3 domain having one of

5 the amino acid sequences shown in Tables 4, 15, and 16, and an antibody having a  $V_H$   
6 CDR3 domain having one of the amino acid sequences shown in Tables 13 and 14.

1 11. The antibody of claim 1, wherein said antibody expressed by any of  
2 the clones listed in Table 16.

1 12. The antibody of claim 1, wherein said antibody is an Fab.

1 13. The antibody of claim 1, wherein said antibody is an  $(Fab')_2$ .

1 14. The antibody of claim 1, wherein said antibody  $(sFv')_2$ .

1 15. The antibody of claim 14, wherein said  $(Sfv')_2$  is a fusion protein  
2 of two sFv' fragments.

1 16. The antibody of claim 1, wherein said antibody is C6.5 Fab.

1 17. The antibody of claim 1, wherein said antibody is C6.5  $(Fab')_2$ .

1 18. The antibody of claim 1, wherein said antibody is C6.5  $(sFv')_2$ .

1 19. The antibody of claim 1, wherein said antibody has a  $K_d$  ranging  
2 from about  $1.6 \times 10^{-8}$  M to  $1.0 \times 10^{-11}$  M in SK-BR-3 using a Scatchard assay or against  
3 purified c-erbB-2 by surface plasmon resonance in a BIAcore.

1 20. The antibody of claim 19, wherein said  $K_d$  is about  $1.6 \times 10^{-8}$  M.  
2

1 21. A nucleic acid encoding a human C6 antibody that specifically  
2 binds to c-erbB-2.

1 22. The nucleic acid of claim 21, wherein said C6 antibody binds to  
2 SK-BR-3 cells with a  $K_d$  less than about  $1.6 \times 10^{-8}$  as determined using a scatchard assay.

1           23.    The nucleic acid of claim 21, wherein said nucleic acid encodes an  
2 an antibody selected from the group consisting of an antibody having a 1V<sub>L</sub> domain  
3 containing one of the amino acid sequences shown in Table 10, an antibody having a V<sub>H</sub>  
4 domain containing one of the amino acid sequences shown in Table 12, an antibody  
5 having a V<sub>L</sub> CDR3 domain containing one of the amino acid sequences shown in Tables  
6 4, 15, and 16, and an antibody having a V<sub>H</sub> CDR3 domain containing one of the amino  
7 acid sequences shown in Tables 13 and 14.

1           24.    The nucleic acid of claim 21, wherein said nucleic acid encodes the  
2 variable light (V<sub>L</sub>) chain of C6.5.

1           25.    The nucleic acid of claim 21, wherein said nucleic acid encodes the  
2 variable heavy (V<sub>H</sub>) chain of C6.5.

1           26.    The nucleic acid of claim 21, wherein said nucleic acid encodes  
2 C6.5.

1           27.    The nucleic acid of claim 21, wherein said nucleic acid encodes the  
2 the amino acid of a C6.5 antibody and conservative amino acid substitutions of said C6.5  
3 antibody.

1           28.    A cell comprising a recombinant nucleic acid that encodes a human  
2 antibody that specifically binds c-erbB-2, wherein said antibody is a C6 antibody.

1           29.    A chimeric molecule that specifically binds a tumor cell bearing c-  
2 erbB-2, said chimeric molecule comprising an effector molecule attached to a human C6  
3 antibody that specifically binds c-erbB-2.

1           30.    The chimeric molecule of claim 28, wherein said C6 antibody is a  
2 single chain Fv (sFv).

1           31.    The chimeric molecule of claim 28, wherein said effector molecule  
2 is selected from the group consisting of a cytotoxin, a label, a radionuclide, a drug, a  
3 liposome, a ligand, and an antibody.

1           32.    The chimeric molecule of claim 28, wherein said effector molecule  
2 is a *Pseudomonas* exotoxin.

1           33.    The chimeric molecule of claim 28, wherein said chimeric molecule  
2 is a fusion protein.

1           34.    A method for making a C6 antibody, said method comprising:  
2           i)    providing a phage library presenting a C6.5 variable heavy ( $V_H$ )  
3 chain and a multiplicity of human variable light ( $V_L$ ) chains;  
4           ii)   panning said phage library on c-erbB-2; and  
5           iii)   isolating phage that specifically bind said c-erbB-2.

1           35.    The method of claim 33, further comprising:  
2           iv)   providing a phage library presenting a the variable light chain ( $V_L$ )  
3 of the phage isolated in step iii and a multiplicity of human variable heavy ( $V_H$ )  
4 chains;  
5           v)    panning said phage library on immobilized c-erbB-2; and  
6           vi)   isolating phage that specifically bind said c-erbB-2.

1           36.    A method for making a C6 antibody, said method comprising:  
2           i)    providing a phage library presenting a C6.5 variable light ( $V_L$ )  
3 chain and a multiplicity of human variable heavy ( $V_H$ ) chains;  
4           ii)   panning said phage library on immobilized c-erbB-2; and  
5           iii)   isolating phage that specifically bind said c-erbB-2.

1           37.    A method for making a C6 antibody, said method comprising:  
2           i)    providing a phage library presenting a C6.5 variable light ( $V_L$ ) and  
3 a C6.5 variable heavy chain encoded by a nucleic acid variable in the sequence  
4 encoding the CDRs such that each phage display a different CDR;

- 1                   ii)    panning said phage library on c-erbB-2; and  
2                   iii)   isolating phage that specifically bind said c-erbB-2.

1                   38.    A method for impairing growth of tumor cells bearing c-erbB-2,  
2   said method comprising contacting said tumor with a chimeric molecule comprising a  
3   cytotoxin attached to a human C6 antibody that specifically binds c-erbB-2.

1                   39.    A method for detecting tumor cells bearing c-erbB-2, said method  
2   comprising contacting said tumor with a chimeric molecule comprising a label attached to  
3   a human C6 antibody that specifically binds c-erbB-2.

1                   40.    A polypeptide comprising one or more of the complementarity  
2   determining regions (CDRs) whose amino acid sequence contains a CDR sequence  
3   selected from the group consisting of the CDRs listed in Tables 4, 10, 12, 13, 14, 15,  
4   and 16.

1                   41.    A nucleic acid molecule comprising a nucleotide sequence  
2   encoding a single chain polypeptide that exhibits the antibody-binding specificity of a  
3   human C6 antibody, said polypeptide comprising:

4                   a)    a first polypeptide domain, comprising an amino acid sequence that  
5   is the binding portion of a variable region of a heavy chain of a human C6 antibody;

6                   b)    a second polypeptide domain, comprising an amino acid sequence  
7   that is the binding portion of a variable region of a light chain of a human C6 antibody;  
8   and  
9

10                  c)    at least one polypeptide linkers comprising an amino acid sequence  
11   spanning the distance between the C-terminus of one of the first or second domains and  
12   the N-terminus of the other, whereby said linker joins the first and second polypeptide  
13   domains into a single chain polypeptide.

14                  42.    A polypeptide which exhibits immunological binding properties of  
15   a human C6 antibody, said polypeptide comprising first and second domains connected  
16   by a linker moiety, wherein:

- 1           a)     the first domain comprises at least one amino acid sequence that is  
2 a CDR derived from a heavy chain of a human C6 antibody; and  
3           b)     the second domain comprises at least one amino acid sequence that  
4 is a CDR derived from a light chain of a human C6 antibody.

1           43.    The polypeptide of claim 42, wherein the first domain comprises a  
2 a heavy chain of a human C6 antibody.

1           44.    The polypeptide of claim 42, wherein the second domain  
2 comprises a light chain of a human C6 antibody.

1           45.    An expression cassette, comprising:  
2           a)     the nucleic acid molecule of claim 41; and  
3           b)     a control sequence operably linked to the nucleic molecule and  
4 capable of directing the expression thereof.

1           46.    An expression cassette, comprising:  
2           a)     the nucleic acid molecule of claim 41; and  
3           b)     a control sequence operably linked to the nucleic  
4 molecule and capable of directing the expression thereof.

1           47.    An expression cassette, comprising:  
2           a)     the nucleic acid molecule of claim 41; and  
3           b)     a control sequence operably linked to the nucleic  
4 molecule and capable of directing the expression thereof.

1           48.    A method of inducing the production of a polypeptide, comprising:  
2           a)     introducing the expression cassette of claim 47 into a host cell  
3 whereby the cassette is compatible with the host cell and replicates in the host cell;  
4           b)     growing the host cell whereby the polypeptide is expressed; and  
5           c)     isolating the polypeptide.

1           49.    A method of inducing the production of a polypeptide, comprising:

- 1                   a)     introducing the expression cassette of claim 47 into a host cell  
2     whereby the cassette is compatible with the host cell and replicates in the host cell;  
3                   b)     growing the host cell whereby the polypeptide is expressed; and  
4                   c)     isolating the polypeptide.

1                   50.     A method of inducing the production of a polypeptide, comprising:

- 2                   a)     introducing the expression cassette of claim 47 into a host cell  
3     whereby the cassette is compatible with the host cell and replicates in the host cell;  
4                   b)     growing the host cell whereby the polypeptide is  
5     expressed; and  
6                   c)     isolating the polypeptide.

**NOVEL HIGH AFFINITY HUMAN ANTIBODIES TO TUMOR  
ANTIGENS**

**ABSTRACT OF THE DISCLOSURE**

This invention provides for novel human antibodies that specifically bind to c-erbB-2. The antibodies may be used alone or as components of chimeric molecules that specifically target and deliver effector molecules to cells overexpressing c-erbB-2.

# Figure 1

10 20 30 40  
\* \* \* \* \*  
CAG GTG CAG CTG TTG CAG TCT GGG GCA GAG TTG AAA AAA CCC GGG GAG  
Gln Val Gln Leu Leu Gln Ser Gly Ala Glu Leu Lys Lys Pro Gly Glu>  
\_a\_a\_a\_a\_a\_\_TRANSLATION OF ERB6 [A]\_a\_a\_a\_a\_a\_\_>

50 60 70 80 90  
\* \* \* \* \*  
TCT CTG AAG ATC TCC TGT AAG GGT TCT GGA TAC AGC TTT ACC AGC TAC  
Ser Leu Lys Ile Ser Cys Lys Gly Ser Gly Tyr Ser Phe Thr Ser Tyr>  
\_a\_a\_a\_a\_a\_\_TRANSLATION OF ERB6 [A]\_a\_a\_a\_a\_a\_\_>

100 110 120 130 140  
\* \* \* \* \*  
TGG ATC GCC TGG GTG CGC CAG ATG CCC GGG AAA GGC CTG GAG TAC ATG  
Trp Ile Ala Trp Val Arg Gln Met Pro Gly Lys Gly Leu Glu Tyr Met>  
\_a\_a\_a\_a\_a\_\_TRANSLATION OF ERB6 [A]\_a\_a\_a\_a\_a\_\_>

150 160 170 180 190  
\* \* \* \* \*  
GGG CTC ATC TAT CCT GGT GAC TCT GAC ACC AAA TAC AGC CCG TCC TTC  
Gly Leu Ile Tyr Pro Gly Asp Ser Asp Thr Lys Tyr Ser Pro Ser Phe>  
\_a\_a\_a\_a\_a\_\_TRANSLATION OF ERB6 [A]\_a\_a\_a\_a\_a\_\_>

200 210 220 230 240  
\* \* \* \* \*  
CAA GGC CAG GTC ACC ATC TCA GTC GAC AAG TCC GTC AGC ACT GCC TAC  
Gln Gly Gln Val Thr Ile Ser Val Asp Lys Ser Val Ser Thr Ala Tyr>  
\_a\_a\_a\_a\_a\_\_TRANSLATION OF ERB6 [A]\_a\_a\_a\_a\_a\_\_>

250 260 270 280  
\* \* \* \* \*  
TTG CAA TGG AGC AGT CTG AAG CCC TCG GAC AGC GCC GTG TAT TTT TGT  
Leu Gln Trp Ser Ser Leu Lys Pro Ser Asp Ser Ala Val Tyr Phe Cys>  
\_a\_a\_a\_a\_a\_\_TRANSLATION OF ERB6 [A]\_a\_a\_a\_a\_a\_\_>

290 300 310 320 330  
\* \* \* \* \*  
GCG AGA CAT GAC GTG GGA TAT TGC AGT AGT TCC AAC TGC GCA AAG TGG  
Ala Arg His Asp Val Gly Tyr Cys Ser Ser Ser Asn Cys Ala Lys Trp>  
\_a\_a\_a\_a\_a\_\_TRANSLATION OF ERB6 [A]\_a\_a\_a\_a\_a\_\_>

340 350 360 370 380  
\* \* \* \* \*  
CCT GAA TAC TTC CAG CAT TGG GGC CAG GGC ACC CTG GTC ACC GTC TCC  
Pro Glu Tyr Phe Gln His Trp Gly Gln Gly Thr Leu Val Thr Val Ser>  
\_a\_a\_a\_a\_a\_\_TRANSLATION OF ERB6 [A]\_a\_a\_a\_a\_a\_\_>

390 400 410 420 430  
\* \* \* \* \*  
TCA GGT GGA GGC GGT TCA GGC GGA GGT GGC TCT GGC GGT GGC GGA TCG  
Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Ser>  
\_a\_a\_a\_a\_a\_\_TRANSLATION OF ERB6 [A]\_a\_a\_a\_a\_a\_\_>

440 450 460 470 480  
\* \* \* \* \*  
CAG TCT GTG TTG ACG CAG CCG CCC TCA GTG TCT GCG GCC CCA GGA CAG  
Gln Ser Val Leu Thr Gln Pro Pro Ser Val Ser Ala Ala Pro Gly Gln>  
\_a\_a\_a\_a\_a\_\_TRANSLATION OF ERB6 [A]\_a\_a\_a\_a\_a\_\_>



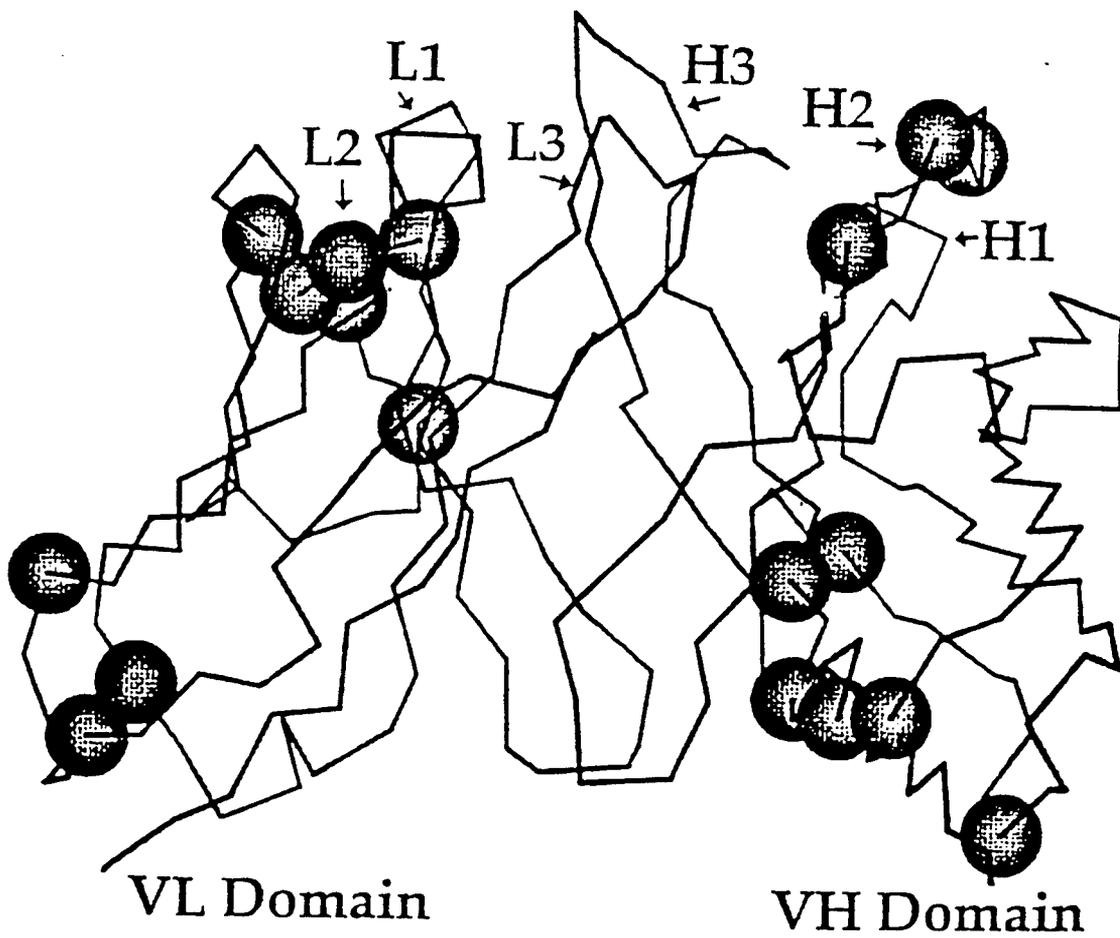


Figure 2

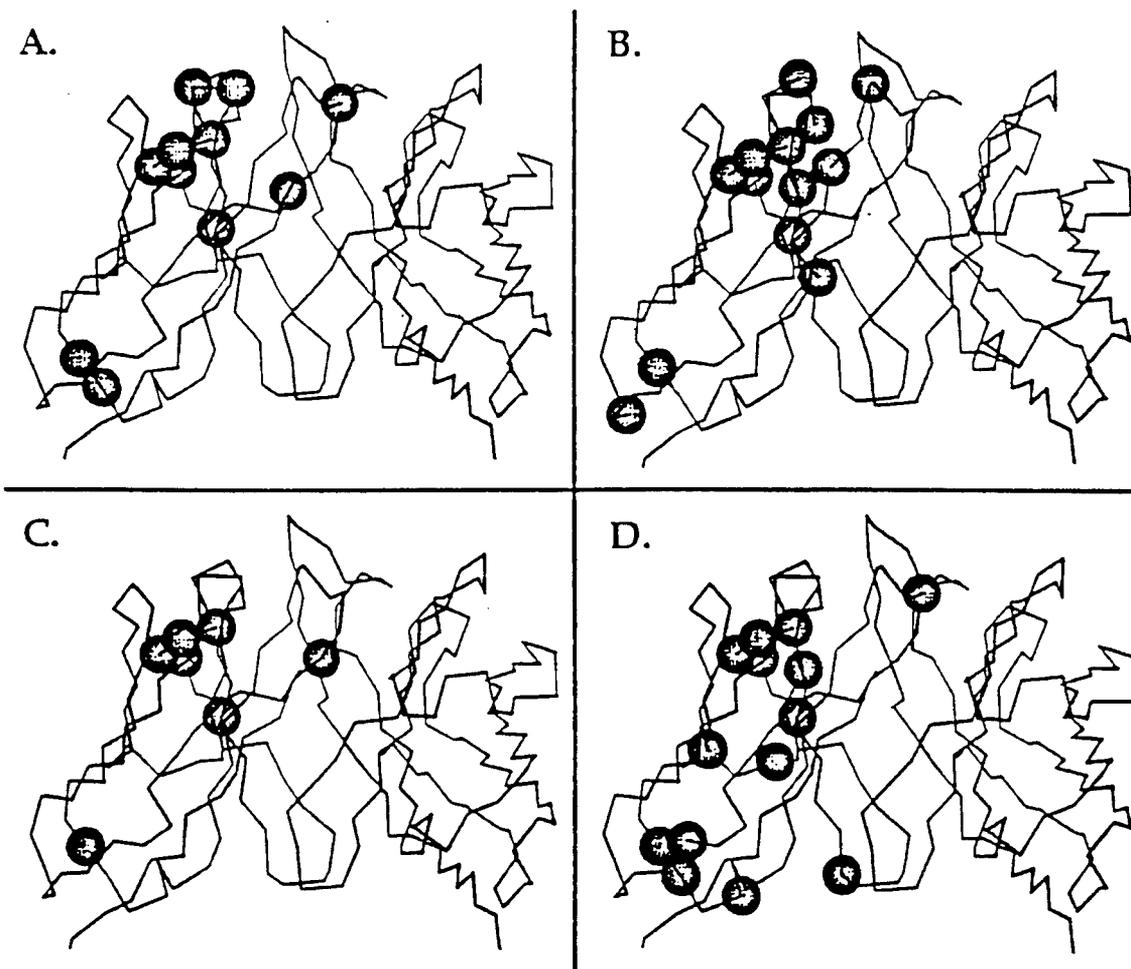


Figure 3

**C6.5 DIABODY**  
**72 hour biodistribution in SK-OV-3**  
**tumor-bearing scid mice**

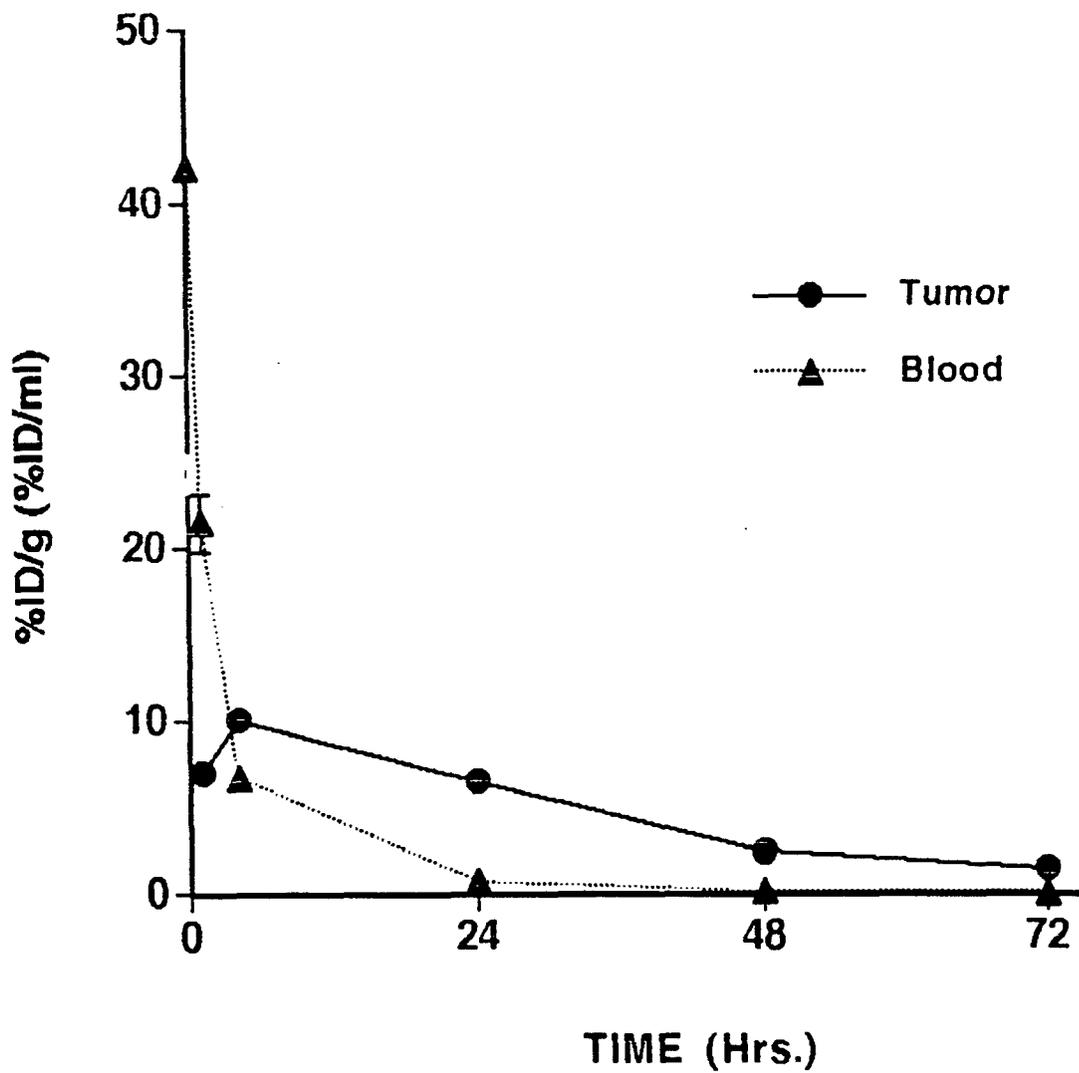


Figure 4



DEPARTMENT OF THE ARMY  
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND  
504 SCOTT STREET  
FORT DETRICK, MARYLAND 21702-5012

REPLY TO  
ATTENTION OF:

MCMR-RMI-S (70-1y)

6 JUN 2001

MEMORANDUM FOR Administrator, Defense Technical Information  
Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir,  
VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports. Request the limited distribution statement for reports on the enclosed list be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

A handwritten signature in black ink, appearing to read "Phyllis M. Rinehart", written over a printed name and title.

PHYLLIS M. RINEHART  
Deputy Chief of Staff for  
Information Management

Encl

Reports to be changed to "Approved for public release;  
distribution unlimited"

<u>Grant Number</u>	<u>Accession Document Number</u>
DAMD17-94-J-4147	ADB221256
DAMD17-93-C-3098	ADB231640
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DAMD17-94-J-4499	ADB258779
DAMD17-94-J-4437	ADB258772
DAMD17-94-J-4437	ADB249591
DAMD17-94-J-4437	ADB233377
DAMD17-94-J-4437	ADB221789
DAMD17-96-1-6092	ADB231798
DAMD17-96-1-6092	ADB239339
DAMD17-96-1-6092	ADB253632
DAMD17-96-1-6092	ADB261420
DAMD17-95-C-5078	ADB232058
DAMD17-95-C-5078	ADB232057
DAMD17-95-C-5078	ADB242387
DAMD17-95-C-5078	ADB253038
DAMD17-95-C-5078	ADB261561
DAMD17-94-J-4433	ADB221274
DAMD17-94-J-4433	ADB236087
DAMD17-94-J-4433	ADB254499
DAMD17-94-J-4413	ADB232293
DAMD17-94-J-4413	ADB240900