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This report describes the results of year 1 of a 4 year grant to develop high affinity human antibody fragments to novel breast tumor antigens. To produce human antibody fragments which bind to novel tumor antigens, we have created a very large (7.0×10^9 member) single chain Fv (scFv) phage antibody library. This library contains multiple antibody fragments to every antigen used for selection. In future work, this library will be selected on breast tumor cell lines to isolate tumor specific antibody fragments. 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 an scFv (C6.5) which binds to the breast tumor antigen c-erbB-2. The affinity of C6.5 was increased 100 fold to a K_d of 1.6×10^{-10} 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.

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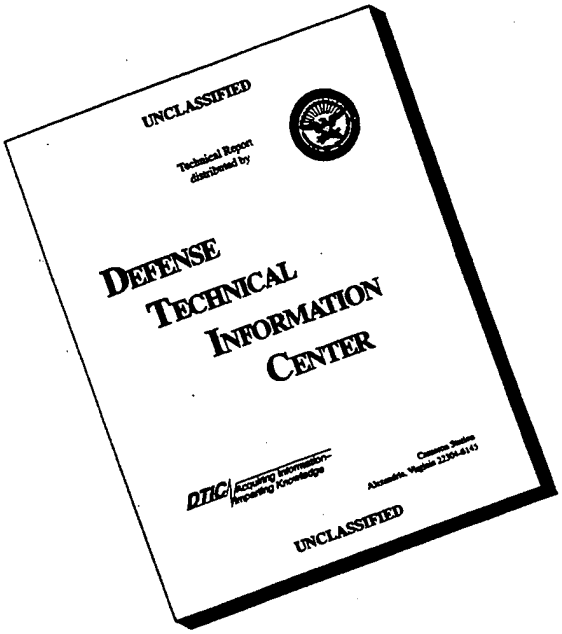

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1. Introduction

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 and 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 1.0×10^{-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 $(Fab')_2$ 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_H and V_L) 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 scFvs with slower dissociation rate constants or by creating dimeric scFvs (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 uM 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 repertories, based on the sequence of a binding scFv, are created and expressed on the surface of phage. Higher affinity scFvs 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_H or V_L 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 phenyloxazolone (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 scFvs 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 first year of this 4 year grant, we would create a large Fab phage antibody library and screen the pre-existing smaller scFv phage antibody library on breast tumor cell lines.

2. Body of report

Work during the first year of the grant focused on creation of a large phage antibody library (technical objective 1.3.2), and screening of the smaller scFv phage antibody library (technical objective 1.3.1) on breast tumor cell lines. For reasons described in section 2.2 below, no tumor specific binding scFv were isolated when the smaller scFv library was selected 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 1.0×10^{-6} M to 1.6×10^{-10} M. The best binders represent the highest affinity antibodies ever produced to a tumor antigen. Higher affinity scFv result in greater quantitative delivery of radiolabelled scFv to c-erbB-2 expressing SK-OV-3 tumors in scid mice.

2.1 Creation of a large scFv phage antibody library

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 (7.0×10^{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 5.0×10^{-8} to 1.0×10^{-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 3.0×10^7 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_κ, C_λ, and IgM primers, and 1st strand cDNA synthesized. 1st strand cDNA was used as a template for PCR amplification of the V_H, V_κ and V_λ gene repertoires. The V_H gene repertoires were cloned into the vector pUC119Sfi-Not as NcoI-NotI fragments, to create a library of 3.2×10^8 members. The library was diverse by PCR fingerprinting. Single chain linker DNA was spliced onto the V_κ and V_λ gene repertoires using PCR and the repertoire cloned as an XhoI-NotI fragment into the vector pHENIXscFv to create a library of 1.6×10^6 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 7.0×10^9 members. The library was diverse as determined by BstN1 fingerprinting.

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 6 and 14 scFv isolated per antigen. This compares favorably to results obtained from the smaller scFv library (1 to a few binders obtained against only 70% of antigens used for selection). Binding scFv were obtained after only 3 rounds of selection, compared to 4 to 5 rounds required with the smaller library. This suggests the affinities are better, but measurements are pending.

Table 1. Specificities and umber of binders isolated from a 7.0×10^9 member scFv phage antibody library.

Antigen	Number of binders
fibroblast growth factor receptor	8
vascular endothelial growth factor	6
bone morphogenic protein receptor	9
activin receptor type 1	11
activin receptor type 2	9
cytochrome b5	6
c-erbB-2	10
α-bungarotoxin	15

2.2 Screening of a 3.0×10^7 member scFv phage antibody library on breast tumor cell lines

A 3.0×10^7 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 then spend time optimizing selections using this library, we focused on production of a much larger scFv phage antibody library (see above). 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.

2.3. 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

We hypothesize that phage display provides the possibility of producing antibodies with affinities that cannot be produced using conventional hybridoma technology. Increased affinity should result in greater quantitative delivery of antibody to the tumor.

Phage display has proven effective as a technique to increase antibody fragment affinity. Mutant phage antibody libraries are created and higher affinity antibody fragments selected (24, 27-31). From a single mutant phage antibody library, affinity can be increased approximately 3 to 5 fold. Despite the successes, many questions remain on how best to create higher affinity antibodies. During the initial year, 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 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 1.6×10^{-8} M and k_{off} of 6.3×10^{-3} s^{-1} (25). Isolation and characterization of C6.5 is described briefly below and in detail in appendix 1, manuscript #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.

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

Human scFvs which bound to c-erbB-2 ECD were isolated by selecting the non-immune human scFv phage antibody library (described in section 2.3 above) on c-erbB-2 extracellular domain immobilized on polystyrene. After five rounds of selection, 45/96 clones analyzed produced scFv which bound c-erb-B2 by ELISA. Restriction fragments analysis and DNA sequencing revealed the presence of two unique human scFvs, C4 and C6.5 . Both of these scFvs 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-erb-B2 expressed on cells, and thus this scFv was selected for further characterization.

2.3.2. 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 hexa-histidine 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 100 different scFv using this technique (see below and manuscripts in appendix 2, 3, and the patent application in appendix 4 for examples).

2.3.3. 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 1.0×10^2 to 5.0×10^6 and k_{off} in the range 1.0×10^{-1} to 1.0×10^{-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} M. We have previously used surface plasmon resonance to determine scFv affinity (22, 24) and have found that affinities measured in this manner correlate well with affinities measured in solution by fluorescence quench titration (J. D. Marks and M. Malmqvist, unpublished data).

2.3.4. 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, 1995, appendix 1). The K_d of 1.6×10^{-8} M determined by BIAcore is in close agreement to the K_d determined by Scatchard analysis after radioiodination (2.0×10^{-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 % 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 scFv (11). The K_d of 741F8 was also measured by BIAcore and agreed reasonably well with the value determined by Scatchard analysis (Table 2).

Table 2 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 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.

	741F8	C6.5
Kd (BIAcore)	2.6×10^{-8} M	1.6×10^{-8} M
Kd (Scatchard)	5.4×10^{-8} M	2.1×10^{-8} M
Kon (BIAcore)	2.4×10^{5} $M^{-1}s^{-1}$	4.0×10^{5} $M^{-1}s^{-1}$
Koff (BIAcore)	6.4×10^{-3} s^{-1}	6.3×10^{-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	5.6a
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

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 sFv 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.

2.3.5. Optimization of conditions for selecting higher affinity monomeric scFv

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., in press, 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_H domain of one scFv molecule pairing with the V_L 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_H and V_L domains to dissociate (41). 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^{-8} M) (43-45). 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 β-sheets which make up the V_H-V_L 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 β-strands in the interface (Table 3 and figure 3, appendix 2). The fourth has an insertion in one of the interface β-strands. In 3 of these scFv, the mutations occur in V_L 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 (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_d 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_d, and less than the phage concentration, was used in the latter rounds of selection (Table 2, appendix 2). 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 (1.0×10^{-11} M) (Table 2, appendix 2). In the case of V_H shuffling, higher affinity binders were only obtained at the lowest antigen concentration (1.0×10^{-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.

2.3.6. Development of a technique for monitoring stringency of selections

As described in section 2.3.5 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 in Schier et al., appendix 2). If the concentrations 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 (Table 3), or the amount (RU) bound (Table 3) correlated linearly with the log of the phage concentration.

Table 3. Correlation between phage antibody titre and BIAcore binding. Monoclonal phage antibody C6ML3-9 (see below) was used as a standard on a CM5 sensor chip coated with 4000 RU of c-erbB-2 ECD. C6ML3-9 phage were prepared by PEG precipitation, diluted to a range of concentrations determined by titration on *E. coli* TG1, the phage injected into the BIAcore, and the binding rates in RU/min calculated from the association part of the sensorgram. The values for total response were taken 15 seconds after the end of the sample injection.

Binding rate RU/min	Response (RU)	Phage concentration (phage/ml)
60	199	2.5×10^{12}
29	118	1.0×10^{12}
15	72	5.0×10^{11}
8	45	2.5×10^{11}

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 4. A C6.5 based mutant phage antibody library was created (C6F) and selected on decreasing concentrations of biotinylated c-erbB-2 ECD in solution (Table 4). The titre of eluted phage decreased the first three rounds of selection but the RU of bound phage went up (Table 4). This indicates positive (and stringent) selection. When the antigen concentration was reduced too low (round 4), 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 5). 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.

Table 4. Results of monitoring selection of phage antibodies using surface plasmon resonance in a BIAcore. A C6.5 scFv phage antibody library (C6F) was created and selected on decreasing concentrations of biotinylated c-erbB-2 ECD in solution. Polyclonal phage were prepared after each round of selection and analyzed for binding to c-erbB-2 using surface plasmon resonance in a BIAcore.

Round of selection	Antigen concentration used for selection ($\times 10^{-12}$ M)	Titre of eluted phage ($\times 10^5$)	Resonance units of phage bound
0	NA	100	48
1	15000	25	219
2	150	6.0	155
3	15	7.0	104
4	1.0	200	23

Table 5. Correlation between binding of polyclonal phage preparations as determined in a BIAcore and percent of ELISA positive clones. A C6.5 scFv phage antibody library (C6F) was created and selected on decreasing concentrations of biotinylated c-erbB-2 ECD in solution. Polyclonal phage were prepared after each round of selection and analyzed for binding to c-erbB-2 using surface plasmon resonance in a BIAcore. The concentration of binding phage was determined from a standard curve constructed from Table 4 and the percent binding phage (BIAcore) determined from the titre of binding phage/titre of the phage preparation. This value is compared to the percent binding phage as determined by ELISA on 96 randomly selected scFv.

Round of selection	Titre phage preparation (phage/ml)	Titre binding phage (BIAcore)	Percent binding phage (BIAcore)	Percent binding phage (ELISA)
0	5.0×10^{12}	2.0×10^{11}	5	2
1	1.1×10^{13}	3.2×10^{12}	29	54
2	5.5×10^{12}	2.0×10^{12}	39	76
3	7.0×10^{12}	1.0×10^{12}	14	16
4	3.5×10^{12}	2.0×10^{11}	5	0

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.

2.3.7. Development of a technique for estimating affinity of unpurified scFv for antigen (c-erbB-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_{50} 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_{off}, measurement of k_{off} should identify higher affinity scFv. k_{off} 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_{off} is independent of concentration. The value of k_{off} for periplasmic and purified sFv is in close agreement (Table 6).

Table 6. Comparison of k_{off} determined on sFv in bacterial periplasm and after purification by IMAC and gel filtration.	scFv	k_{off} (s^{-1})
	C6-5 periplasm	5.7×10^{-3}
	C6-5 purified	6.3×10^{-3}
	C6-5ala3 periplasm	9.3×10^{-3}
	C6-5ala3 purified	1.5×10^{-2}
	C6-5ala10 periplasm	3.7×10^{-3}
	C6-5ala10 purified	4.1×10^{-3}

Significance:

A technique has been developed which allows ranking of mutant scFv by k_{off}, 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_L and V_HCDR3 randomization below).

2.3.8 Development of a technique to determine optimal elution conditions to use during phage antibody selections

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 8 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 7). The most effective solutions were 50 mM and 100 mM HCl. 2.6 M $MgCl_2$, 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 $MgCl_2$; 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 (Tables 8 and 9). The highest affinity scFv were obtained when eluting with solutions demonstrated by BIAcore to be most efficacious in removing bound phage (100 mM HCl).

Table 7. Effects of different elution solutions on removing bound phage from c-erbB-2 ECD coupled to a CM5 sensor chip, as determined by surface plasmon resonance in a BIAcore. Polyclonal anti-c-erbB-2 phage (2.2×10^{12} transforming units/ml) were allowed to bind to 1400 resonance units (RU) of c-erbB-2 ECD coupled to a CM5 sensor chip in a BIAcore. After 5 minutes of association, running buffer (hepes buffered saline) was applied and the amount (RU) of bound phage determined (t=0). After 2 minutes of dissociation, 1 of the 6 eluents was applied for 2 minutes at a flow rate of 10 µl/min. The amount of phage that remained bound was determined 2 minutes later (t=6 minutes), and the percentage of the bound phage that was eluted was expressed as RU phage bound (t=6 min)/RU phage bound (t=0). Major differences were observed in the efficacy of the eluents in removing bound phage.

Eluent	RU phage bound (t=0)	RU phage bound (t=6 min)	% bound phage eluted
Hepes buffered saline	190	150	21
2.6 M $MgCl_2$	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 8. Effect of eluent on the affinities and binding kinetics of selected scFv. Values represent the mean of the 8 highest affinity scFv resulting from each selection.

Eluent	K_d ($\times 10^{-9}$ M)	k_{on} ($\times 10^5$ s^{-1} M^{-1})	k_{off} ($\times$ 10-3 s-1)
No elution	5.39 ± 0.73	4.64 ± 0.35	2.49 ± 0.41
1 µM c-erbB-2	5.99 ± 1.12	5.09 ± 0.27	2.58 ± .47
2.6 M $MgCl_2$	3.30 ± 0.45†	5.05 ± 0.43	1.58 ± .14
100 mM TEA	2.65 ± 0.35*	4.78 ± 0.39	1.27 ± .20
10 mM HCl	6.09 ± 1.29	5.72 ± 0.30	3.46 ± .80
50 mM HCl	2.60 ± 0.40*	6.38 ± 1.02	1.54 ± .19
100 mM HCl	2.52 ± 0.46*	5.99 ± 0.37	1.40 ± .20

* = $p<0.05$ compared to no elution, 1 µM c-erbB-2, and 10 mM HCl.
† = $p<0.05$ compared to 10 mM HCl.

Table 9. Effect of elution conditions on sequences, affinities and binding kinetics of individual scFv. Eight scFv with the slowest k_{off} were identified by BIAcore screening of 20 ELISA positive clones from each selection. The 8 were subcloned, purified, k_{on}, k_{off}, and K_d determined, and the V_λ DNA sequenced.

Clone	V_L CDR3 sequence	K_d ($\times 10^{-9}$ M)	k_{on} ($\times 10^5$ s^{-1} M^{-1})	k_{off} ($\times$ 10-3 s-1)
C6.5	AAWDDSLSGWV	16.0		6.3
NO ELUTION				
C6ML3-5 (4)	----Y------	3.7	5.1	1.9
C6ML3-17	-S--YYR----	5.0	3.4	1.7
C6ML3-1	----Y--W---	6.7	3.0	2.0
C6ML3-22	----A------	8.3	4.3	3.6
C6ML3-26	------R----	8.3	6.0	5.0
1 µM c-erbB-2				
C6ML3-5 (5)	----Y------	3.7	5.1	1.9
C6ML3-17	-S--YYR----	5.0	3.4	1.7
C6ML3-25 (2)	----NRH----	7.4	5.9	4.4
2.6 M $MgCl_2$				
C6ML3-12	----Y-R----	1.6	4.5	0.72
C6ML3-15	----RP-W---	2.2	5.9	1.3
C6ML3-7 (2)	----YAV----	2.6	6.5	1.7
C6ML3-5 (2)	----Y------	3.7	5.1	1.9
C6ML3-16	-S--Y-R----	3.8	5.5	2.1
C6ML3-17	-S--YYR----	5.0	3.4	1.7

100 mM TEA				
C6ML3-19	-S--RP-W---	1.5	6.6	1.0
C6ML3-12 (2)	----Y-R----	1.6	4.5	0.72
C6ML3-18	-S--A--W---	2.4	2.6	0.62
C6ML3-20	---EQ--W---	3.0	4.7	1.4
C6ML3-5 (3)	----Y------	3.7	5.1	1.9
10 mM HCl				
C6ML3-23	-S--H--W---	1.5	6.7	1.0
C6ML3-7	----YAV----	2.6	6.5	1.7
C6ML3-5	----Y------	3.7	5.1	1.9
C6ML3-21	----Y-Q----	4.5	4.9	2.2
C6ML3-25	----NRH----	7.4	5.9	4.4
C6ML3-22	----A------	8.3	4.3	3.6
C6ML3-26	------R----	8.3	6.0	5.0
C6ML3-24	----EQIF----	12.4	6.4	7.9
50 mM HCl				
C6ML3-12 (2)	----Y-R----	1.6	4.5	0.72
C6ML3-29	----GT-W---	1.7	12.9	2.2
C6ML3-28	-S--YA-----	2.5	6.8	1.7
C6ML3-7 (2)	----YAV----	2.6	6.5	1.7
C6ML3-6	-S--Y------	3.2	5.9	1.9
C6ML3-17	-S--YYR----	5.0	3.4	1.7
100 mM HCl				
C6ML3-9	-S--YT-----	1.0	7.6	0.76
C6ML3-14 (2)	-----P-W---	1.1	7.0	0.77
C6ML3-15	----RP-W---	2.2	5.9	1.3
C6ML3-5 (4)	----Y------	3.7	5.1	1.9

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.

2.3.9 Identification of structural and functional residues in the V_H and V_L domains of scFv

Since it is difficult to make libraries greater than 10^7 to 10^8 clones, decisions must be made as to which residues to diversify, 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 (50). 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, 51, 52). For the rest of the residues, a decrease in entropy accounts for most of the enthalpy decrease, resulting in no net effect on affinity (51, 52). In many instances, 'repulsive contacts' are also made, which can cost up to several kcal (51). 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. (53)). The limits of the loops are defined structurally as lying outside of the β-sheet (54, 55) and these limits are slightly different than the complementarity determining regions (CDRs) defined by Kabat on the basis of sequence hypervariability (56). 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 (54-56). 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 (57). 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.

To determine the utility of PM, the technique was used to increase the affinity of C6.5 (25). 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 x 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 appendix 3, Schier et al, in press Gene.

The V_λ 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 (58). L1 consists of 9 residues, L2 of 3 residues, and L3 of 8 residues (54). The V_H domain of C6.5 is derived from the DP73 germline gene of the $V_H 5$ family (59) and could be modeled using the three dimensional structure of the V_H domain of NC41 (60). H1 consists of 7 residues, H2 of 6 residues, and H3 of 17 residues (55). 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 (53). 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 (54, 55). 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, (56)) 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 (61), a library of 1.0 x 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 x 10^{-8} M and decreasing to 1.0 x 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_{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_{off} slower than the parental scFv, while after four rounds of selection, 10/20 scFv (50%) had a slower k_{off}. All 13 scFv with a slower k_{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 10). 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 (2.4×10^{-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 (2.4×10^{-9} M) compares favorably to the affinity of murine antibodies produced against the same antigen using conventional hybridoma technology (11, 62).

Table 10. Kinetics of selected scFv subjected to parsimonious mutagenesis.

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

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 11). 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_HY52) 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_H domain. In the $V\lambda$ domain, residues I28, G29, W91, and D92 are present in both C6.5 and KOL (58), 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 (54). In the model of C6.5, $V\lambda$G29, $V\lambda$G95b, and V_HG53 are in turns and $V\lambda$W91 and $V\lambda$W96 pack against the V_H domain at the V_H-V_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.

Table 11. Deduced protein sequences of CDRs of C6.5 and C6.5 mutants from the parsimonious mutagenesis phage antibody library.

	V_HCDR2	V_λCDR1	V_λCDR3
Residue number	5 5 5 6	2 2 2 3	8 9 9
	0-2a3------0-----	4--7ab8-0----	9-----5ab6-
C6.5	LIYPGDSDTKYSPSFQG	SGSSSNIGNNYVS	AAWDDSLSGWV
3rd round selection			
PM1	----------S--	-------MD----	-----T-----
PM2	F----------	--------K----	----E-WT---
PM3	S----NY----------	----------	----Y------
4th round selection			
PM4	----------	----------	----------
PM5	------YG---------	----------T--	----Y------
PM6	------YG---------	--------K----	----Y------
PM7	----------	----------	----H------
PM8	---A---A---------	----------	----Y------
PM9	----------	--------K----	------Y----
PM10	----------	--------D----	----A-QY---
PM11	----------	--------S----	-----A-----
PM12	----------	F----------	----E------
PM13	------YG---------	--------K----	----Y------

2.3.10 Comparison of techniques for introducing mutations into antibody V_H and V_L genes

In the work that follows, we sought to identify the most effective location in the antibody V-genes to introduce mutations for the purpose of producing higher affinity antibodies. Methods examined included chain shuffling and site directed mutagenesis of the CDRs.

Results of experiments to increase the affinity of C6.5 by chain shuffling

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) (23, 63). This method relies on the natural diversity of V-genes present normally in the human repertoire. The approach has been successfully used to increase the affinity of a non-immune human scFv which bound the hapten phenyloxazolone 300 fold from 3.0 x 10^{-7} M to 1.0 x 10^{-9} M by sequentially shuffling the rearranged V_L gene and the V_H gene segment (the wild type V_HCDR3 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_H and V_L genes of gp120 binding Fabs resulted in Fabs of "similar apparent binding constants" (64, 65). 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_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 6 fold to 2.5 x 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 appendix 2, Schier et al., in press, Journal of Molecular Biology.

To alter the affinity of C6.5, a mutant scFv gene repertoire was created containing the V_H gene of C6.5 and a human V_L gene repertoire (light chain shuffling). The scFv gene repertoire was cloned into the phage display vector pHEN-1 and after transformation a library of 2×10^5 transformants was obtained. For heavy chain shuffling, the C6.5 V_H CDR3 and light chain were cloned into a vector containing a human V_H gene repertoire to create a phage antibody library of 1×10^6 transformants. After selection, a single higher affinity light chain shuffled scFv (C6L1) was identified. C6L1 had a K_d 6 times lower than C6.5. After selection, 2 higher affinity heavy chain shuffled scFv were identified. C6H2 had a K_d 5 times lower than C6.5. 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. 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. 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.

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_H and V_L shuffled chains were combined. This is unexpected, typically the effect of mutations are additive (28, 66). The reason for the lack of additivity is unclear, but suggests that a sequential approach to chain shuffling (24) may be more prudent.

2.3.11 Results of experiments to increase the affinity of C6-5 by site directed mutagenesis of V_LCDR3 and V_HCDR3.

As described previously in the section on parsimonious mutagenesis, the majority of antigen contacting amino acid side chains are located in the antigen binding loops, three in the V_H (H1, H2, and H3) and three in the V_L (L1, L2, and L3. These residues contribute the majority of binding energetics responsible for antibody affinity for antigen. In other molecules, mutating amino acids which are at the ligand interface has been shown to be an effective means of increasing the affinity of one protein molecule for its binding partner. Since we cannot mutate all these residues simultaneously, the question we sought to answer was, which residues give the greatest increase in affinity. The majority of the binding energetics in antibody-antigen interactions result from a few residues located at the center of the binding pocket. Most of these residues are located in L3 and H3. We hypothesized that mutating neighboring residues located in H3 and L3 would be the most effective means to increase antibody affinity. Initial experiments involved diversification of L3, given its shorter length. Thus to increase the affinity of C6.5 for c-erbB-2, 7 amino acids in L3 were partially randomized by synthesizing a 'doped' oligonucleotide in which the wild type nucleotide occurred with a frequency of 70%, and the other three nucleotides at a frequency of 10%. The oligonucleotide was used to amplify the remainder of the C6.5 scFv gene using PCR. The resulting scFv gene repertoire was cloned into pCANTAB5E (Pharmacia) to create a phage antibody library of 1×10^7 transformants. Selection of the C6.5 mutant V_L CDR3 library 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. scFv 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 scFvs with the slowest k_{offs} from the 3rd and 4th round of selection were sequenced and each unique scFv subcloned into pUC119 Sfi-NotmycHis. scFv was expressed, purified by IMAC and gel filtration,

and scFv affinity and binding kinetics determined by BIAcore (Table 12). Mutant scFv were identified with 16 fold increased affinity for c-erbB-2.

Table 12. Amino acid sequence, affinity, and kinetics of binding of C6.5 light chain CDR3 mutants. Affinity and kinetics of binding were determined on purified, gel filtered scFv in a BIAcore.

scFv clone	L3 sequence	K_d (M)	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})
C6.5	AAWDDSLSGWV	1.6×10^{-8}	4.0×10^{5}	6.3×10^{-3}
3rd Round of selection				
C6ML3-5	----Y------	3.2×10^{-9}	5.9×10^{5}	1.9×10^{-3}
C6ML3-2	----H------	2.8×10^{-9}	7.1×10^{5}	2.0×10^{-3}
C6ML3-6	-S--Y------	3.2×10^{-9}	5.9×10^{5}	1.9×10^{-3}
C6ML3-1	----Y--W---	6.7×10^{-9}	3.0×10^{5}	2.0×10^{-3}
C6ML3-3	-T--YA-----	4.3×10^{-9}	4.6×10^{5}	2.0×10^{-3}
C6ML3-7	----YAV----	2.6×10^{-9}	6.5×10^{5}	1.7×10^{-3}
C6ML3-4	-S-EY--W---	3.5×10^{-9}	4.0×10^{5}	1.4×10^{-3}
4th Round of selection				
C6ML3-12	----Y-R----	1.6×10^{-9}	4.5×10^{5}	7.2×10^{-4}
C6ML3-9	-S--YT-----	1.0×10^{-9}	7.6×10^{5}	7.6×10^{-4}
C6ML3-10	---E-PWY---	2.3×10^{-9}	6.1×10^{5}	1.4×10^{-3}
C6ML3-11	----YA-W---	3.6×10^{-9}	6.1×10^{5}	2.2×10^{-3}
C6ML3-13	----AT-W---	2.4×10^{-9}	8.7×10^{5}	2.1×10^{-3}
C6ML3-8	----HLRW---	2.6×10^{-9}	6.5×10^{5}	1.7×10^{-3}
C6ML3-35	-----P-W---	1.0×10^{-9}	7.7×10^{5}	7.7×10^{-4}

2.3.12. Results of experiments to increase the affinity of C6.5 by site directed mutagenesis of V_HCDR3.

H3 typically contributes the greatest number of antigen contacting residues and in the case of C6.5 is also remarkably long (20 amino acids, figure 1). Since it is not possible to scan sequences this long completely, we used homology modeling to identify which residues within CDR3 to mutate. The C6.5 VHCDR3 is homologous to the VHCDR3 of the antibody Fv KOL (figure 1).

Figure 1. Amino acid sequence of the H3 of C6.5 aligned with the H3 of the antibody KOL.

```
                    1         10        20
C6.5 VH CDR3        HDV GYCSSSNCAKWPEYFQH
KOL VH CDR3         DGGHGFCSSASCFG PDY
```

Analysis of H3 in the crystallographic structure of the antibody KOL shows a 4 residue loop protruding from the binding pocket stabilized by a disulfide at the base of the loop (58). It was hypothesized that these residues were likely to be solvent accessible and important for antigen binding. Starting with C6ML3-9 ($K_d = 1.0 \times 10^{-9}$ M), we randomized the amino acid sequence in C6.5 located between the 2 cysteine residues (SSSN), using the nucleotides NNS. The library was created and selected as described above, and clones characterized after 4 rounds of selection. The results are shown in Table 13 below. The highest affinity mutant (C6MH3-B1) had a K_d 6 fold lower than the starting clone C6ML3-9, and 100 fold lower than C6.5.

Table 13. Affinities, binding kinetics, and V_HCDR3 sequences of scFv resulting from mutation of a portion of the V_HCDR3.

Clone name	CDR3 sequence	K_d (M)	Koff (s^{-1})
C6.5	HDVGYCSSSNCAKWPEYFQH	1.6×10^{-8}	6.3×10^{-3}
C6MH3-B1	------TDRT----------	1.6×10^{-10}	6.7×10^{-5}
C6MH3-B15	------ESSR----------	7.7×10^{-10}	2.9×10^{-4}
C6MH3-B11	------SDRS----------	2.2×10^{-10}	2.3×10^{-4}
C6MH3-B9	------KTAA----------	8.7×10^{-10}	3.3×10^{-4}
C6MH3-B8	------*TER----------	7.2×10^{-10}	2.9×10^{-4}

C6MH3-B5	------TDAT----------	5.3×10^{-10}	2.3×10^{-4}
C6MH3-B2	------TDPR----------	3.1×10^{-9}	3.1×10^{-4}
C6MH3-B39	------TDPT----------	3.2×10^{-10}	1.9×10^{-4}
C6MH3-B25	------LTTR----------	3.6×10^{-10}	1.9×10^{-4}
C6MH3-B21	------TTPL----------	7.3×10^{-10}	2.4×10^{-4}
C6MH3-B20	------SPAR----------	8.7×10^{-10}	1.6×10^{-4}
C6MH3-B16	------ADVR----------	3.1×10^{-10}	1.8×10^{-4}

Significance:

Evolution of the center of the antibody combining site (V_LCDR3 and V_HCDR3) resulted in an antibody with 100 fold higher affinity than wild type. Introduction of mutations into this region appears to be the most effective means to increase antibody affinity.

2.3.13. 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_d ranging from 1.0×10^{-6} M to 1.0×10^{-9} 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. These experiments were conducted in the laboratory of Dr. Lou Weiner. Cell surface retention assays demonstrate that scFv with a slower k_{off} are retained to a greater extent than scFv with more rapid k_{off}. For example, the three fold slower k_{off} of C6L1 correlated with a three fold 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).

Preliminary *in vivo* binding and biodistribution results also demonstrate a positive effect of affinity on biodistribution. For example, tumor retention of 0.14 %ID/g was observed for ^{125}I-C6.Gly4Ala (K_d=1.0 x 10^{-7} M), and 0.78 %ID/g for ^{125}I-C6.VLA1 (K_d=2.5 x 10^{-9} M; p = 0.00056). In a confirmatory study, the 24 hour tumor retention of C6.5 was 0.67 %ID/g, while that of C6.L1 was 1.13 %ID/g (p = 0.048).

Significance:

These results demonstrate that selective tumor retention of sFv molecules correlates with their affinity properties and that further improvements in affinity will be required to achieve substantial improvements in selective tumor retention by sFv. With further increases in affinity, additional improvements in tumor retention should be observed.

3. Conclusions

1. The binding properties of scFv in small (3 x 107 member) phage antibody libraries are not adequate to allow their use to identify novel tumor antigens. Therefore, a much larger (7.0 x 10^9 member) phage antibody library was produced. This library contains multiple scFv capable of binding any antigen, presumably with higher affinity. In future work, we will use this library to identify novel tumor specific antigens, and to produce scFv against these antigens.
2. The selection technique (immobilized or soluble antigen) and eluent affect the affinities of antibodies isolated from mutant phage antibody libraries. Successful isolation of higher affinity antibodies requires the use of soluble antigen in limiting concentrations. As antibody affinity increases, more stringent solutions are required to elute the highest affinity antibodies. Techniques were developed to select, elute, and identify rare higher affinity mutant scFv amidst a background of lower affinity scFv.
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. Mutation of these residues at a higher frequency results in even higher affinity antibodies.

4. Introduction of mutations into the center of the antibody combining site (V_H and V_L CDR3) was the most effective means of increasing antibody affinity. Using this approach, we were able to increase affinity of a breast tumor targeting antibody 100 fold to a $K_d = 1.6 \times 10^{-10}$ M. This represents the highest affinity tumor targeting antibody produced.
5. Increased affinity correlated with greater retention of antibody on cells in vitro, and in tumors in scid mice. While the actual amounts retained are not dramatic, we have yet to study the highest affinity mutants. We expect the quantitative retention to increase significantly as the koff decreases below 10^{-4} s^{-1}, giving a $t_{1/2}$ on cells longer than the elimination $t_{1/2}$ from the body (2.5 hours).

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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.

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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 1.6×10^{-8} M and to c-erbB-2 on SK-OV-3 cells with a K_d of 2.0×10^{-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_H, immunoglobulin heavy chain variable region; V_L, immunoglobulin light chain variable region; ECD, extracellualr domain; PBS, phosphate-buffered saline; IPTG, isopropyl-β-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_6 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 (3×10^7 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 μg/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, 7.0×10^{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 μg/ml ampicillin and 25 μg/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 NaH_2PO_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 μg/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 $A_{600\,nm}$ of 0.8–0.9. After incubation for 30 min at 37°C, bacteria were plated on TYE plates containing 100 μg/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] ($A_{600\,nm} \sim 0.8$) were infected with 100 μl 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 microtire plate wells containing 150 μl of 2 × TY containing 100 μg/ml ampicillin and 0.1% glucose. The bacteria were grown to an $A_{600\,nm} \sim 1.0$, 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 microtiter plates (Falcon 3912) were coated overnight at 4°C with 10 μg/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 μl 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 $A_{405\,nm}$ 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 μg/ml of bovine serum albumin, hen egg white lysozyme, bovine glutamyltranspeptidase, c-erbB-2 ECD, VCS M13 (3.5×10^{12}/ml) and casein (0.5%). For ELISA with biotinylated c-erbB-2 ECD, microtiter plates (Immunolon 4, 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 1 h at 37°C and incubated with 50 μl biotinylated c-erbB-2 extracellular domain (5 μg/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 *Nco*I and *Not*I, and the sFv DNA purified on a 1.5% agarose gel. C6.5 sFv DNA was ligated into pUC119Sfi1/Not1Hismyc digested with *Nco*I and *Not*I and the ligation mixture used to transform electrocompetent *E. coli* HB2151. For expression, 200 ml of 2 × TY media containing 100 μg/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 $A_{600\,nm}$ 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 × *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 × *g* for 15 min. and the 'periplasmic fraction' cleared by centrifugation at 30 000 × *g* for 20 min. The supernatant was 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-μm filter.

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, 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 $A_{280\,nm}$ 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 μg/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 continuous flow of 5 μl/min. k_{on} was determined from a plot of $(\ln(dR/dt))/t$ vs. concentration [16]. k_{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 μg of radioiodinated sFv was added to 5×10^6 SK-OV-3 cells in the presence of increasing concentrations of unlabelled sFv from the same preparation. After a 30-min incubation at 20°C, the samples were washed with PBS at 4°C and centrifuged at 500 × *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}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. Ten μg of CT (Sigma, St. Louis, MO) was added per 100 μg of protein and the resulting mixture was incubated for 3 min at room temperature. The reaction was quenched by the addition of 10 μg of sodium metabisulfite (Sigma) per 100 μg 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}I-C6.5 sFv and typically about 1.0 mCi/mg for the ^{125}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}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 μg of either sFv with 2×10^6 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 × *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 (2.5×10^6) 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}I-C6.5 sFv was diluted in PBS to a concentration of 0.2 mg/ml and each mouse was given 100 μl, containing 20 μg 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 μg (100 μl) of ^{131}I-C6.5. At 24 h after injection, the mice were euthanized by asphyxiation with 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 Vλ1 family (Table 1). The V_L gene appears to be derived from two different germline genes (HUMLV122 and DPL 5) suggesting the occurence 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 Vλ3 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	QVQLVESGGGLVQPGGSLRLSCAASGFTFS	SYEMN	WVRQAPGKGLEWVS	YISSSGSTIYYADSVKG	RFTISRDNAKNSLYLQMNSLRAEDTAVYYCAR	DLGGYSYGYVGLDY	WGQGTLVTVSS
DP58	E----------	-----	----------	----------	----------		
C6.5	QVQLLQSGAELKKPGESLKISCKGSGYSFT	SYWIA	WVRQMPGKGLEYMG	LIYPGDSDTKYSPSFQG	QVTISVDKSVSTAYLQWSSLKPSDSAVYFCAR	HDVGYCSSSNCAKWPEYFQH	WGQGTLVTVSS
DP73	E---V-----V----------	----G	----------W--	I--------R-------	-----A---I----------A--T-M-Y---		

Light chains

	Framework 1	CDR1	Framework 2	CDR2	Framework 3	CDR3	Framework 4
C4.1	SELTQDPAVSVALGQTVRITC	QGDSLRSYYAS	WYQQKPGQAPVLVIY	GKNNRPS	GIPDRFSGSSSGNIASLTITGAQAEDEADYYC	NSRDSSGNPYWV	FGGGTKVTVLG
IGLV3S1	-S----------	----------	----------	-------	----------T----------	--------H V-	
C6.5	QSVLTQPPSVSAAPGQKVTISC	SGSSSNIGNNYVS	WYQQLPGTAPKLLIY	GHTNRPA	GVPDRFSGSKSGTSASLAISGFRSEDEADYYC	AAWDDSLSG WV	FGGGTKLTVLG
HUMLV122	----------	----------	----------	DNKK--S	-I----------T-G-T-LQTG-------	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)	2.6×10^{-8} M	1.6×10^{-8} M
K_d (Scatchard)	5.4×10^{-8} M	2.1×10^{-8} M
k_{on} (BIAcore)	2.4×10^5 $M^{-1}s^{-1}$	4.0×10^5 $M^{-1}s^{-1}$
k_{off} (BIAcore)	6.4×10^{-3} s^{-1}	6.3×10^{-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[a]
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.

[a]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 (1.6×10^{-8} M) agreed closely to the value determined by Scatchard (2.0×10^{-8} M) (Table 2). Kinetic analysis by BIAcore indicated that C6.5 had a rapid on-rate (k_{on} 4.0×10^5 M^{-1} s^{-1}) and a rapid off-rate (k_{off} 6.3×10^{-3} s^{-1})

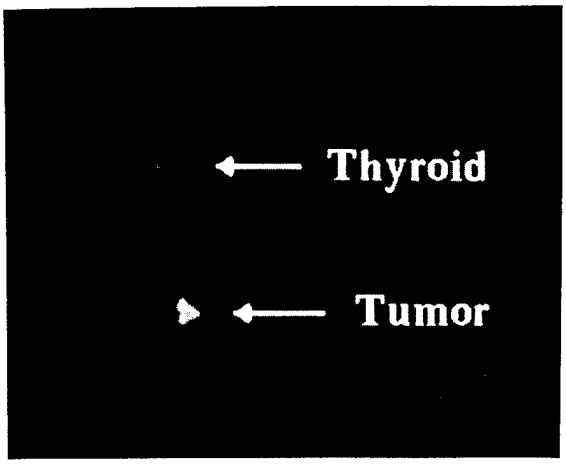

Fig. 1. Radioimmunoimaging of subcutaneous SK-OV-3 tumors in C.B17/ICR-scid mice by ^{131}I-C6.5. Gamma camera images were obtained at 24 h after the i.v. administration of 100 μg (140 μCi) of C6.5. Image acquisition was terminated when 100 000 counts were acquired.

(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 adsorbtion 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., in press.

Isolation of high affinity monomeric human anti-c-erbB-2 single chain Fv using affinity driven selection[a]

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Key Words: c-erbB-2; single chain Fv; phage antibody libraries; affinity maturation, chain shuffling

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 β-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 NaH_2PO_4, 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; t.u., transducing units V_κ, immunoglobulin kappa light chain variable region; V_λ, immunoglobulin lambda light chain variable region; V_L, immunoglobulin light chain variable region; V_H, immunoglobulin heavy chain variable region.

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Abstract

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 applications. For hapten antigens, higher affinity scFv can be isolated from phage antibody libraries where the V_H and V_L gene 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 1.6×10^{-8} M. The affinity of the parental scFv was increased 6 fold ($K_d = 2.5 \times 10^{-9}$ M) by light chain shuffling and 5 fold ($K_d = 3.1 \times 10^{-9}$ M) by heavy chain shuffling, values comparable to 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 ($K_d = 1.1 \times 10^{-8}$ 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.

Introduction

Despite the demonstration of tumor specific and tumor associated antigens, the use of monoclonal antibodies for therapy of cancers has not yielded consistent beneficial responses (reviewed in (Riethmuller *et al.*, 1993)). The disappointing results can partially be attributed to limitations of monoclonal IgG antibodies and limitations of the hybridoma technology used to generate them. 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 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 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, 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; Griffiths *et al.*, 1993; Marks *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 almost invariably express 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) which binds specifically to the extracellular domain (ECD) of the tumor antigen c-erbB-2 (McCartney *et al.*, 1995) with a K_d of 1.6×10^{-8} M and k_{off} of 6.3×10^{-3} s^{-1} (Schier *et al.*, 1995). Biodistribution studies in scid mice demonstrate high tumor:normal organ ratios and excellent tumor visualization, 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; Hawkins *et al.*, 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 successfully used to increase the affinity of a non-immune human scFv which bound the hapten phenyloxazolone 300 fold from 3.0×10^{-7} M to 1.0×10^{-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 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_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 6 fold to 2.5×10^{-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 3 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_κ and V_λ gene repertoires were cloned into the phage display vector pHEN1-Vλ3 (Hoogenboom & Winter, 1992) to create a 4.5 x 10^6 member library (pHEN1-$V_L$rep, figure 1a). The resulting library contains DNA encoding the single chain linker sequence $(G_4S)_3$, and cloning sites for inserting the rearranged V_H gene from a binding scFv as an NcoI-XhoI fragment (figure 1b). Polymerase chain reaction (PCR) screening of pHEN1-$V_L$rep revealed that 95% of clones analyzed had full length insert and a diverse BstNI restriction pattern. To shuffle the light chain of C6.5, the rearranged C6.5 V_H gene was cloned into pHEN1-$V_L$rep as an NcoI-XhoI fragment (figure 1b). After transformation, a library of 2.0 x 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 which 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_H gene segment (excluding the V_H CDR3) was shuffled. The rationale is that V_H CDR3 results from splicing of three gene segments (V_H, D, and J), is the most genetically diverse part of the rearranged V_H gene, and in non-immune repertoires it is unlikely that many similar V_H CDR3s exist. Since V_H CDR3 contributes a disproportionate number of amino acid residues which contact antigen, shuffling the rearranged V_H gene would result in a library containing few binding scFv. For heavy chain shuffling, human V_H gene segment repertoires (framework1 (FR1) to FR3) were cloned into the phage display vector pHEN1 (Hoogenboom *et al.*, 1991) (figure 1c). The resulting library contains a human V_H gene segment repertoire and cloning sites for inserting the V_H CDR3, FR4, single chain linker, and rearranged light chain gene from a binding scFv as a BssHII-NotI fragment (figure 1d). Three heavy chain gene repertoires were created

(pHEN1-V_H1rep, pHEN1-V_H3rep, and pHEN1-V_H5rep), each enriched for V_H1, V_H3, or V_H5 gene segments by using PCR primers designed to anneal to the consensus sequence of the 3' end of V_H1, V_H3, or V_H5 FR3 (Tomlinson *et al.*, 1992). These primers also introduced a BssHII site at the end of FR3, without changing the amino acid sequence typically observed at these residues. Libraries were constructed from these three V_H gene families since they make up over 95% of the V_H genes of non-immune scFv (Marks *et al.*, 1992; Griffiths *et al.*, 1993; Marks *et al.*, 1993). Libraries of 5.0×10^5 clones for pHEN1-V_H1rep, 1.0×10^6 clones for pHEN1-V_H3rep and 1.5×10^6 clones for pHEN1-V_H5rep were obtained. Analysis of 50 clones from each library indicated that greater than 80% of the clones had insert, and the libraries were diverse by the BstNI restriction pattern (Marks *et al.*, 1991). Three heavy chain shuffled libraries were made by cloning the C6.5 V_H CDR3, FR4, linker, and light chain gene into the previously created V_H1, V_H3, or V_H5 gene segment library vectors using the BssHII and NotI restriction sites (figure 1d). After transformation, libraries of $1.0\text{-}2.0 \times 10^6$ clones were obtained. PCR screening revealed that 100% of clones analyzed had full length insert and a diverse BstNI restriction pattern. Prior to selection, 20/92 clones selected at random from the V_H5 shuffled library expressed scFv which bound c-erbB-2. 0/92 clones selected at random from the V_H1 or V_H3 shuffled library expressed scFv which 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 2nd and the 3rd round of selection were analyzed for binding to recombinant c-erbB-2 ECD by enzyme linked immunosorbent assay (ELISA). After the 3rd 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}s less than

the IC_{50} of the parental scFv were sequenced and four unique DNA sequences identified (Table 3). All 4 rearranged $V\lambda$ genes were putatively derived from the same $V\lambda$ family ($V\lambda1$) 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/NotmycHis. 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). 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}s, 3 of the 4 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 (1.1×10^{-8} M) than C6.5. k_{off} of the dimeric scFv fraction was 3 to 4 fold 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.

As a result of these observations, we hypothesized that selection on immobilized antigen favored the isolation of dimeric scFv which 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_n/R_0) vs t, the presence of multiple k_{off} could be detected, indicative of the presence of mixtures of monomers, dimers, or higher molecular weight 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 4 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, 13/90 clones were positive for c-erbB-2 binding by ELISA after the 4th 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 4th 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_λ gene of C6L1 was derived from the same germline gene (DPL5, (Williams & Winter, 1993)) as the parental scFv, but had 9 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 6 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 three fold slower k_{off} correlated with a three fold 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 4 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-2 binding by ELISA after the 4th 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 4th 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 segment 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 7 and 9 amino acids respectively. C6H1 also had an opal stop codon (TGA) in the heavy chain CDR1 and 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 3 fold higher affinity for c-erbB-2 than C6.5 and C6H2 had 5 fold higher affinity than C6.5 (Table 4). The increased affinity of C6H1 (5.9×10^{-9} M) was due to a faster k_{on}, whereas the increased affinity of C6H2 (3.1×10^{-9} M) was due to both a faster k_{on} and slower k_{off} (Table 4).

The expression level of C6H1 (opal stop codon) was reduced 100 fold compared to C6.5 (10 mg of purified C6.5/L of *E. coli* culture compared to 50 μg/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) (figure 3). KOL was selected as the model because it has a V_λ 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 4 light chain shuffled scFv which formed mixtures of monomer and dimer had mutations in residues which form the β-sheet that packs on the V_H domain (Table 3 and figure 3). In contrast, scFv which did not form dimers (C6L1, C6H1, and C6H2) did not have mutations located in the V_H-V_L interface, except for 2 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 which 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 nonimmune phage antibody library. The scFv were produced without any immunization, are entirely human in sequence, and the affinities (2.5×10^{-9} M and 3.1×10^{-9} M) compare favorably to the affinities of hybridoma antibodies 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 which 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_H domain of one scFv molecule pairing with the V_L 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 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. 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 (Deng *et al.*, 1995). 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.

Experimental results suggest 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^{-8} 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 β-sheets which 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 4 light chain shuffled mutants which dimerize have substitutions in amino acids which comprise at least one of the β-strands in the interface. The fourth has an insertion in one of the interface β-strands. In

3 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 (1.0×10^{-11} M). In the case of V_H shuffling, higher affinity binders were only obtained at the lowest antigen concentration (1.0×10^{-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 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 (Hawkins *et al.*, 1993), 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.

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) (Friguet *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 vs 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 which did not have a higher affinity. In the case of heavy chain shuffling, where only 1 in 8 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 5 to 6 fold 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 8 fold 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 5 fold 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 5 and 6 fold 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 which 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 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 (2.0×10^{-3} s^{-1}) translates into a theoretical $t_{1/2}$ on the cell surface of less than 10 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 h, reduction of the k_{off} to $<10^{-5}$ s^{-1} ($t_{1/2}$ 18 h) is likely to be required, a value unlikely to be achieved with antibodies produced from hybridomas (Foote & Eisen, 1995). One approach to increase affinity further is to combine mutations which 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 BssHII-NotI 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 1.8×10^8 member scFv phage antibody library in pHEN-1 (Marks *et al.*, 1991). For PCR, 50 ul reactions were prepared containing 10 ng template, 25 pmol back primer (LMB3), 25 pmol forward primer (PVH1FOR1, PVH3FOR1, or PVH5FOR1), 250 µM-dNTPs, 1.5 mM $MgCl_2$, and 0.5 µl (2 units) Taq DNA polymerase (Promega) in the manufacturers 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 30s, 55°C for 30s and 72°C for 30s) 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 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 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, 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 segment repertoire ligated separately into pHEN-1 (Hoogenboom *et al.*, 1991) digested with NotI and NcoI. The ligation mix was purified by extraction with phenol/chloroform, ethanol precipitated, resuspended in 20 µl water, and 2.5 µl samples electroporated (Dower *et al.*, 1988) into 50 µl *E.coli* TG1 (Gibson, 1984). Cells were grown in 1 ml SOC (Sambrook *et al.*, 1990) for 30 min and then plated on TYE (Miller, 1972) media containing 100 µg 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) containing 100 µg ampicillin/ml, 1% glucose (2 x 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 (Gussow & Clackson, 1989) exactly as described in (Marks *et al.*, 1991). The resulting phage libraries were termed pHEN1-V_H1rep, pHEN1-V_H3rep, and pHEN1-V_H5rep.

Three separate C6.5 heavy chain shuffled phage antibody libraries were made from the pHEN1-V_H1rep, pHEN1-V_H3rep, and pHEN1-V_H5rep phage libraries. The C6.5 light chain gene, linker DNA, and V_H 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 BssHII and NotI and ligated into pHEN1-V_H1rep, pHEN1-V_H3rep, and pHEN1-V_H5rep 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 pHEN1-V_λ3 (Hoogenboom and Winter, 1992) containing rearranged human V_κ and V_λ gene repertoires, linker DNA, and cloning sites for inserting a rearranged V_H gene as an NcoI-XhoI fragment. An XhoI 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_κ and V_λ gene repertoire was amplified by PCR from a 1.8×10^8 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 (fdSEQ1). The Back primers were designed to anneal to the first 6 nucleotides of the $(G_4S)_3$ linker and either the J_H1, 2, 6, J_H3, or J_H 4,5 segments respectively. The PCR reaction mixture was purified as described above, digested with XhoI and NotI, gel purified and ligated into pHEN1-$V_\lambda3$ (Hoogenboom and Winter, 1992) digested with XhoI and NotI. Transformation of *E.coli* TG1, PCR screening, and creation of library stocks was as described above. The resulting phage library was termed pHEN1-$V_L$rep.

The light chain shuffled phage antibody library was made from pHEN1-$V_L$rep. The rearranged C6.5 V_H 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 XhoI and NcoI, gel purified and ligated into pHEN-1-$V_L$rep digested with XhoI and NcoI. Transformation of *E.coli* TG1, PCR screening, and creation of library stocks was as described above.

Construction of scFv containing highest affinity V_H and V_L genes obtained by chain shuffling

Two new scFv were made by combining the rearranged V_L gene of the highest affinity light chain shuffled scFv (C6L1) with the rearranged V_H gene of the highest affinity heavy chain shuffled scFv (C6H1 or C6H2). The C6L1 plasmid was digested with NcoI and XhoI to remove the C6.5 V_H gene and gel purified. The rearranged V_H gene of C6H1 or C6H2 was amplified by PCR using the primers LMB3 and PC6VH1FOR, 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.

Preparation of phage

To rescue phagemid particles from the libraries, 10 ml of 2 x TY-AMP-GLU were inoculated with an appropriate volume of bacteria (approximately 50 to 100 µl) from the library stocks to give an A_{600} of 0.3 to 0.5 and grown for 30 min, shaking at 37°C. About 1×10^{12} 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 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 µ filter. The phage preparation consistently resulted in a titre of approximately 10^{13} 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 1 h at 37°C with 2% skimmed milk powder in PBS (2%MPBS) and the selection, washing, and elution were performed exactly as described in (Marks *et al.*, 1991) using phage at a concentration of 5.0×10^{12} 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 3 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 in (Hawkins *et al.*, 1992) but 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 (Pierce). For each round of selection, 1 ml of phage (approximately 10^{13} 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. 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 1 h 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 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 min, rotating on an under-and-over-turntable at room temperature. In S2, 100µl (round 1) or 50 µl (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 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 in (Marks *et al.*, 1991) with the following exception. After overnight

growth and expression at 30°C, 50 µl 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 scFv in the bacterial supernatant. For selection performed on Immunotubes, ELISA plates (Falcon 3912) were incubated with c-erbB-2 ECD (2.5 µg/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 µg/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 (Schier *et al.*, 1995). In both cases, binding of scFv to c-erbB-2 ECD was detected with the mouse monoclonal antibody 9E10 (1 µg/ml), which recognizes the C-terminal peptide tag (Munro & Pelham, 1986), and peroxidase-conjugated anti-mouse Fc antibody (Sigma), as described in (Marks *et al.*, 1991). Selected binders were further characterized by sequencing of the V_H and V_L genes (Sanger *et al.*, 1977). Putative germline gene segment derivation was determined by alignment to the VBASE sequence directory (Tomlinson *et al.*). The clone C6H1 was sequenced in both directions to confirm the presence of an opal (TGA) stop codon in CDR1. Sequence data has been deposited with GenBank, accession numbers U36535-U36559.

Screening of scFv for relative affinity was performed essentially as described in (Friguet *et al.*, 1985). Immunolon 4 ELISA plates (Dynatech) were coated with avidin in PBS (10 µg/ml) at 4°C overnight. Biotinylated c-erbB-2 ECD (5 µg/ml) was added to the wells and incubated for 30 min at room temperature. Bacterial supernatant containing scFv was incubated with varying concentrations of c-erbB-2 (0 to 100 nM) at 4°C for 1 h. The amount of free scFv was then determined by transferring 100 µl of each mixture into the wells of the previously prepared ELISA plate and incubating for 1 h at 4°C. Binding of scFv was detected as under ELISA screening and the IC_{50} calculated as described in (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 induced with isopropylβ-D-thiogalactopyranoside (IPTG) (De Bellis and Schwartz, 1990). Cultures were grown overnight at 25°C, scFv harvested from the periplasm (Breitling *et al.*, 1991), and the periplasmic fraction dialyzed for 24 h 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 μg/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 μl/min. An 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 $MgCl_2$ 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. 200 ml cultures of *E.coli* TG1 harboring one of the C6.5 mutant phagemids were grown, expression of scFv induced with IPTG (De Bellis and Schwartz, 1990), and the culture grown at 25°C overnight. scFv was 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 micron filter.

scFv was purified by IMAC (Hochuli *et al.*, 1988) exactly as described in (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, kinetics, and cell surface retention

The K_d of scFv were determined using surface plasmon resonance in a BIAcore. In a BIAcore flow cell, approximately 1400 RU of c-erbB-2 ECD (90 kDa, McCartney *et al.*, 1995) (25 µg/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 µl/min using a concentration range from 50 to 800 nM. k_{on} was determined from a plot of (ln (dR/dt))/t vs 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 was determined using surface plasmon resonance 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/sec) determined under a constant flow of 30 µl/sec. Over the concentration range of 1.0×10^{-9} M to 1.0×10^{-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 (1.0×10^{-8} M and 2.0×10^{-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. Concentration determined by BIAcore was within 5% of the concentrations determined by A_{280}. 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 5.0×10^{-7} M c-erbB-2 ECD. This was accomplished using the 'kinject' command, resulting in the passage of either HBS or 5.0×10^{-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 bulk refractive index change 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 in (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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Table 1. Sequences of primers used.

LMB3	5'-CAGGAAACAGCTATGAC-3'
fd-seq1	5'-GAATTTTCTGTATGAGG-3'
PHEN-1seq	5'-CTATGCGGCCCCATTCA-3'
Linkseq	5'-CGATCCGCCACCGCCAGAG-3'
PVH1FOR1	5'-TCGCGCGCAGTAATACACGGCCGTGTC-3'
PVH3FOR1	5'-TCGCGCGCAGTAATACACAGCCGTGTCCTC-3'
PVH5FOR1	5'-TCGCGCGCAGTAATACATGGCGGTGTCCGA-3'
PVH1FOR2	5'-GAGTCATTCTCGACTTGCGGCCGCTCGCGCGCAGTAATACACGGCCGTGTC-3'
PVH3FOR2	5'-GAGTCATTCTCGACTTGCGGCCGCTCGCGCGCAGTAATACACAGCCGTGTCCTC-3'
PVH5FOR2	5'-GAGTCATTCTCGACTTGCGGCCGCTCGCGCGCAGTAATACATGGCGGTGTCCGA-3'
PC6VL1BACK	5'-AGCGCCGTGTATTTTTGCGCGCGACATGACGTGGGATATTGC-3'
RJH1/2/6Xho	5'-ACCCTGGTCACCGTCTCGAGTGGTGGA-3'
RJH3Xho	5'-ACAATGGTCACCGTCTCGAGTGGTGGA-3'
RJH4/5Xho	5'-ACCCTGGTCACCGTCTCGAGTGGTGGA-3'
PC6VH1	5'-GAGTCATTCTCGTCTCGAGACGGTGACCAGGGTGCC-3'

Table 2. Frequency of binding scFv and percent of binding scFv with slower k_{off} than C6.5. Binding was determined by ELISA. k_{off} was determined by BIAcore on unpurified scFv in bacterial periplasm.

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, 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=1.0×10^{-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	QSVLTQPPSVSAAPGQKVTISC	SGSSSNIGNNYVS	WYQQLPGTAPKLLIY	GHTNRPA	GVPDRFSGSKSGTSASLAISGFRSEDEADYYC	AAWDDSLSG WV	FGGGTKLTVLG
DPL5[a]	---------A-GT---R-----	--------S---Y	---------------	RNNQ--S	----------L----------	---------	
Light chain shuffled mutants selected on polystyrene adsorbed antigen							
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---------H	DNNK--S	-I---I----------LQ-D-------	-------N- --	----------
Light chain shuffled mutant selected on biotinylated antigen							
C6L1	----------G----W-----	----------	----------	DNNK--S	---------Q----------L----------	--------- --	------V----

CDR, complementarity-determining region; dashes indicate sequence identity. Numbering is according to Kabat (Kabat *et al.*, 1987). Underlined residues are those that form the β-sheet interface that packs on the V_H domain (Chothia *et al.*, 1985). a. The C6.5 V_λ gene is putatively derived from the DPL5 germline gene (Williams & Winter, 1993).

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

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

Table 5. Deduced protein sequences of heavy 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		40 50 60		70 80 abc 90	100	103 100
C6.5	QVQLLQSGAELKKPGESLKISCKGSGYSFT	SYWIA	WVRQMPGKGLEYMG	LIYPGDSDTKYSPSFQG	QVTISVDKSVSTAYLQWSSLKPSDSAVYFCAR	HDVGYCSSSNCAKWPEYFQH	WGQGTLVTVSS
DP73[a]	E---V-----V----------	----G	----------W--	I--------R-------	-----A---I----------A--T-M-Y---		
Heavy Chain shuffled mutants selected on high concentration biotinylated antigen							
C6VHA2	----V---G-M----------L--D--	T----	----------	----------	----------R---T-M-Y---	----------	----------
C6VHB2	----Q---G-M----------L--D--	T----	----------	----------	-----A-E-I-----E-----A--T-M-Y---	----------	----------
C6VHC2	----Q---G-M----------E---S	T----	----------	----------	----------T-------R---T---Y---	----------	----------
C6VHD2	----VE----M----------F--D-S	T----	----------	----------	----------T-M-Y---	----------	----------
C6VHE2	----VE--G-M---R----------L--D--	T----	----------	----------	----------R---T-M-Y---	----------	----------
C6VHF2	----VE--G-M----------L--D-S	T----	----------	----------	----------R---T-M-Y---	----------	----------
C6VHG2	----VE--G-M----------L--D--	T----	----------	----------	----------R---T-M-Y---	----------	----------
C6VHH2	----VE----M----------F--D-S	T----	----------	----------	----------R---T-M-Y---	----------	----------
C6VHA3	--------G-M----------L--D--	T----	----------	----------	----------R---T-M-Y---	----------	----------
C6VHB3	----V---G-M----------L--D-S	T----	----------	----------	-----A-E-I-----E-----A--T-M-Y---	----------	----------
C6VHC3	----Q---G-M----------L--D--	T----	----------	----------	----------R---T-M-Y---	----------	----------
C6VHD3	----Q---G-M----------L--D--	T----	----------	--------IR-----E-	-------R-I-------T---A--T-M-Y---	----------	----------
C6VHE3	----V---G-M---R----------L--D--	T----	----------	----------	----------R---T-M-Y---	----------	----------
C6VHF3	----V-----M----------F--D-S	T----	----------	----------	----------T-M-Y---	----------	----------
C6VHG3	----V-----V--------Q--------D--	T-Y--	----------	I------R-I-------	--R--A----------A--T-M-Y---	----------	----------
C6VHH3	-----E----V-E--Q--------F--D-S	T----	----------	----------	----------T-M-Y---	----------	----------
Heavy Chain shuffled mutants selected on lower concentration biotinylated antigen							
C6H1	----VE----V----------	--*--	----------	----------	----------T-M-Y---	----------	----------
C6H2	----V-----V----------	-----	----------	----A--K-I-------	----------T--T-M-Y---	----------	----------

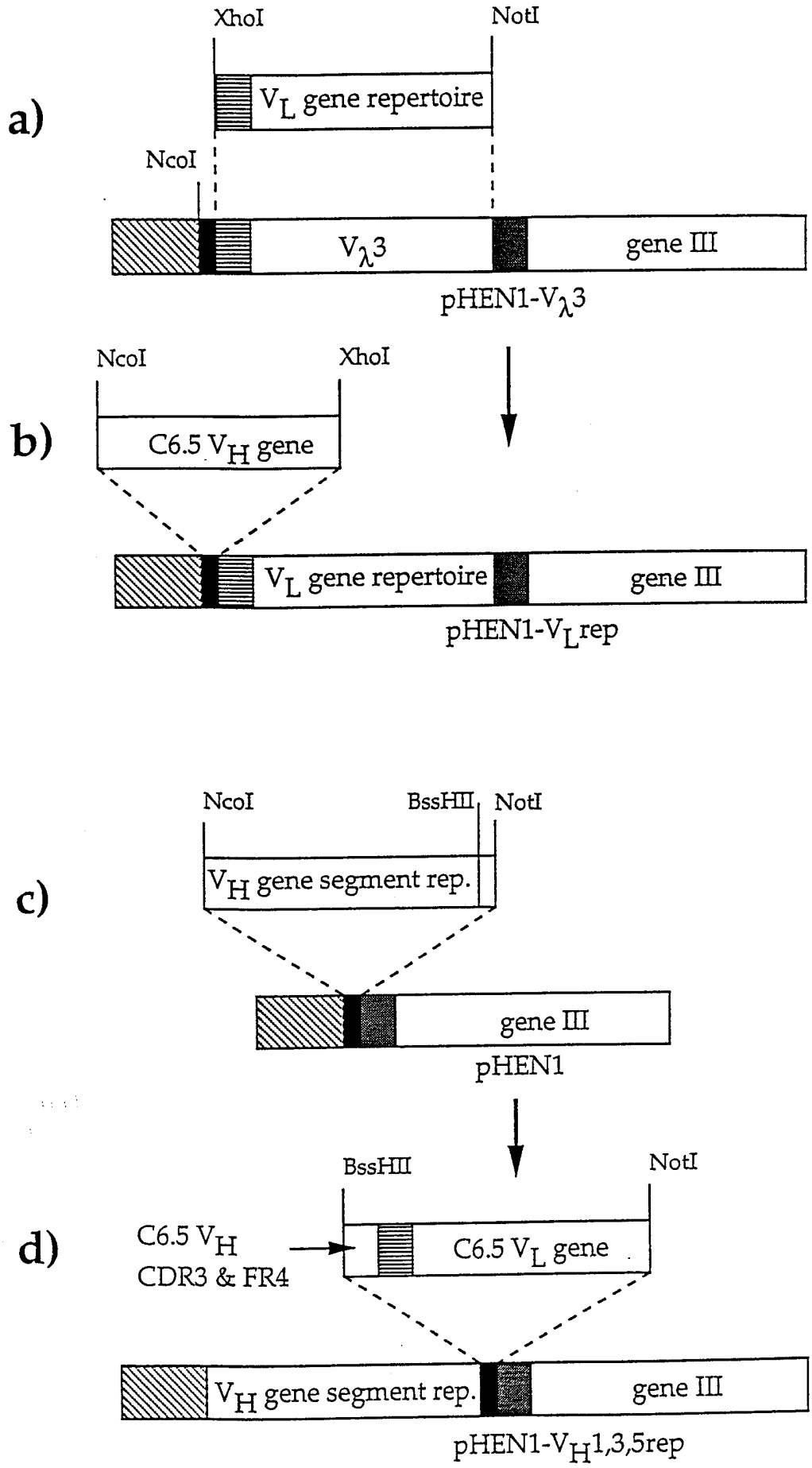
CDR, complementarity-determining region; dashes indicate sequence identity. Numbering is according to Kabat (Kabat *et al.*, 1987). Underlined residues are those that form the β-sheet interface that packs on the V_L domain (Chothia *et al.*, 1985). a. The C6.5 V_H gene is putatively derived from the DP73 germline gene (Tomlinson, *et al.*, 1992).

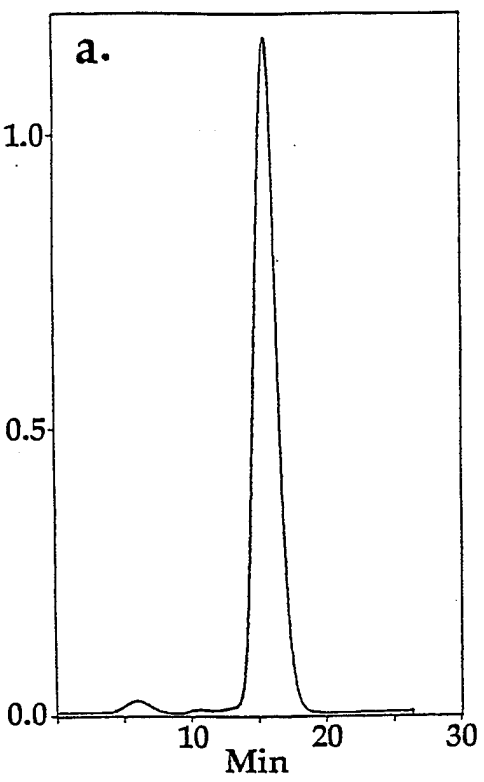
Figure legends

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 $(G_4S)_3$ spliced to the 5′ end (horizontally hatched box). The V_L gene repertoire-single chain linker was cloned as an XhoI-NotI fragment into pHEN1-Vλ3 (Hoogenboom *et al.*, 1992) to create the library vector pHEN1-$V_L$rep. **b) Construction of C6.5 light chain shuffled library.** The rearranged C6.5 V_H gene was cloned as an NcoI-XhoI fragment into pHEN1-$V_L$rep. **c) Construction of human heavy chain libraries for light chain shuffling.** PCR was used to create human V_H1 V_H3, and V_H5 family gene segment repertoires (FR1-FR3, excluding CDR3) containing a BssHII site at the end of FR3. The V_H gene segment repertoires were cloned as NcoI-NotI fragments into pHEN1 (Hoogenboom *et al.*, 1991) to create the library vector pHEN1-V_H1rep, pHEN1-V_H3rep, or pHEN1-V_H5rep. **d) Construction of C6.5 heavy chain shuffled libraries.** The C6.5 V_H CDR3 gene, single chain linker gene, and light chain gene were cloned as an BssHII-NotI fragment into pHEN1-V_H1rep, pHEN1-V_H3rep, or pHEN1-V_H5rep.
(▨ pelB leader sequence, ■ multiple cloning site polylinker, ■ myc peptide tag, ▤ (G4S)3 single chain linker).

Figure 2. Results of gel filtration analysis of C6.5 scFv and chain shuffled mutants. scFv was 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.

Figure 3. Location of mutations in light chain and heavy chain shuffled scFv. Amino acid residues which differ from the residues in C6.5 scFv are indicated as red spheres on the Cα-carbon tracing of the Fv fragment of the immunoglobulin KOL (Marquart *et al.*, 1980). The V_H domain is shown in green and the V_L domain in yellow. Panel a: 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; Panel b, C6VLB; panel c, C6VLD; panel d, C6VLE; panel f, C6VLF. scFv which form mixtures of monomer and dimer (C6VLB, C6VLD, C6VLE, and C6VLF, panels b-e) all have mutations located in the V_H-V_L interface. In contrast scFv which do not form dimers (C6L1, C6H1 (not shown), and C6H2, panel a) do not have mutations located in the V_H-V_L interface, except for 2 conservative mutations located in V_H FR3 of C6H2.


a)
XhoI
NotI
V_L gene repertoire
NcoI
$V_\lambda 3$
gene III
pHEN1-$V_\lambda 3$
b)
NcoI
XhoI
C6.5 V_H gene
V_L gene repertoire
gene III
pHEN1-$V_L$rep
c)
NcoI
BssHII
NotI
V_H gene segment rep.
gene III
pHEN1
d)
BssHII
NotI
C6.5 V_H
CDR3 & FR4
C6.5 V_L gene
V_H gene segment rep.
gene III
pHEN1-V_H1,3,5rep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10
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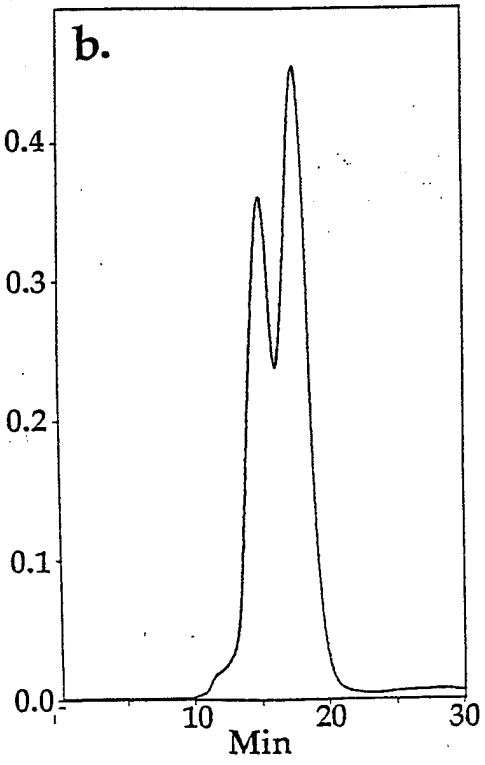
30
Min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0.1
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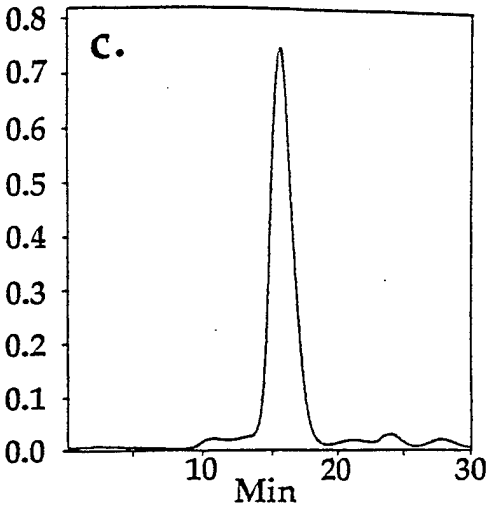
30
Min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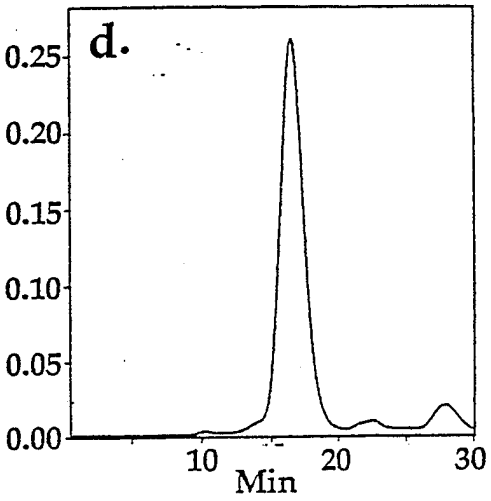
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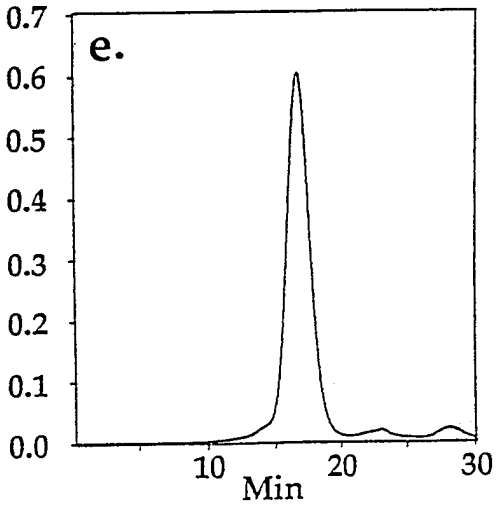
0.10
0.05
0.00
10
20
30
Min


e.
0.7
0.6
0.5
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0.3
0.2
0.1
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Appendix 3

Schier R, Balint RF, McCall A, Apell G, Larrick JW, Marks JD. Identification of functional and structural amino acid residues by parsimonious mutagenesis. Gene, in press.

Identification of functional and structural amino acid residues by parsimonious mutagenesis

Key Words: c-erbB-2, single chain Fv, affinity maturation, random mutagenesis, phage display

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Abbreviations Used: aa, amino acid; CDR, complementarity determining region; 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; 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, nucleotides; PCR, polymerase chain reaction; PM,

parsimonious mutagenesis; scFv, single chain Fv fragment; V_H, immunoglobulin heavy chain variable region; V_λ, immunoglobulin light chain variable region.

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Summary

For *in vitro* evolution of protein function, we previously proposed using parsimonious mutagenesis (PM), a technique where mutagenic oligonucleotides are designed to minimize coding sequence redundancy and limit the number of 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 amino acid residues located in three of the heavy (H) and light (L) chain antigen binding loops (L1, L3, and H2) were simultaneously mutated. After 4 rounds of selection, 50% of scFv had a slower dissociation rate constant (k_{off}) than the parental scFv. The K_d of these scFv ranged from 2 fold (K_d = 7.0 x 10^{-9}M) to 6 fold (K_d = 2.4 x 10^{-9}M) lower than the parental scFv (K_d = 1.6 x 10^{-8} M). In higher affinity scFv, substitutions occurred at 10/19 of the positions, with 21/28 substitutions occurring at only 4 positions, 2 in H2, and 1 each in L1 and L3. Only the wild-type residue was observed at 9/19 residues. Based on a model of C6.5, seven of the nine conserved residues have a structural role in the variable domain, either in maintaining the main chain conformation of the loop, or in packing on the heavy chain variable domain . Two of the conserved residues are solvent exposed, suggesting they may play a critical role in recognition. Thus, PM identified three types of residues: structural residues, functional residues which modulate affinity and functional residues which are critical for recognition. Since the sequence space was not completely sampled, higher affinity scFv could be produced by subjecting functional residues 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.

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 (Foote and Eisen, 1995). The first two limitations have been largely overcome by the display of natural (Marks et al., 1991) and synthetic antibody variable region gene repertoires (Hoogenboom and Winter, 1992) on the surface of phage (McCafferty et al., 1990; Hoogenboom et al., 1991). Human antibody fragments can be recovered from these libraries against virtually any antigen, including haptens, foreign proteins, cell surface antigens, and self-antigens (Marks et al., 1991; Griffiths and Malmqvist, 1993; Marks et al., 1993; Griffiths et al., 1994; Nissim et al., 1994). The affinities of antibodies to protein antigens, however, range from 10^{-6} M 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 antibody fragments isolated from natural, synthetic, or immune phage antibody libraries (Hawkins et al., 1992; Marks et al., 1992; Riechmann and Weill, 1993; Barbas et al., 1994; Schier et al., in press). The sequence of a binding phage antibody is diversified and higher affinity binders selected from the mutant antibody library. Since it is difficult to make libraries greater than 10^7 to 10^8 clones, decisions must be made as to which residues to diversify, 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 (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 residues, 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 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 (Wilson and Stanfield, 1993). The limits of the loops are defined structurally as lying outside of the β-sheet (Chothia and Lesk, 1987; Chothia et al., 1992) and these limits are slightly different than the complementarity determining regions (CDRs) defined by Kabat on the basis of sequence hypervariability (Kabat et al., 1987). 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 (Chothia and Lesk, 1987; Kabat et al., 1987; Chothia et al., 1992). 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 (Balint and Larrick, 1993). 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.

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 antibody 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 6 fold higher affinity for c-erbB-2 (K_d=2.4 x 10^{-9} M) were selected from the library and residues within the loops important for modulation of affinity identified.

Results and Discussion

a. Selection of sites to be mutagenized and doping codons

The V_λ 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 (Marquart et al., 1980). L1 consists of 9 residues, L2 of 3 residues, and L3 of 8 residues (Chothia and Lesk, 1987). The V_H domain of C6.5 is derived from the DP73 germline gene of the $V_H 5$ 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 7 residues, H2 of 6 residues, and H3 of 17 residues (Chothia et al., 1992). 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 (Wilson and Stanfield, 1993). 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 (Chothia and Lesk, 1987; Chothia et al., 1992). 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, (Kabat et al., 1987)) 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. PM-CAD was used to select mutation frequencies, doping codons, and to compute nucleotide mixtures for oligonucleotide synthesis (Balint and Larrick, 1993). 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 (Tables I and II).

b. Construction and characterization of the PM phage antibody library

The PM randomized C6.5 scFv gene repertoire was assembled from three overlapping PCR fragments consisting of a portion of the parental scFv gene and the mutagenized L1, L3, or H2 (figure 1). The N-terminal fragment (PM1) extended from upstream of an SfiI cloning site to ~40 nt beyond the mutagenized region of H2, which was encoded by the downstream primer H2F (Table III and figure I). The second fragment (PM2) extended from the C-terminus of HCDR2 to ~40 nt downstream of 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 ~40 nt downstream of 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 1.0×10^6 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 8.8×10^5. The V_H and V_L genes of 8 unselected scFv were sequenced to determine the frequency and location of mutations in the library (Table VI). Each scFv gene averaged 4 amino acid substitutions in the three regions subjected to PM, with a range of 3 to 6 substitutions. In addition, an

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

c. Selection and characterization of higher affinity scFv

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 4.0×10^{-8} M and decreasing to 1.0×10^{-11} M (Table IV). 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 (Schier et al., in press). 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 (Table IV). The dissociation rate constant (k_{off}) (Karlsson et al., 1991) 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 (Jønsson et al., 1991). After three rounds of selection, 3 of 20 scFv (12%) had a k_{off} slower than the parental scFv, while after four rounds of selection, 10/20 scFv (50%) had a slower k_{off}. All 13 scFv with a slower k_{off} were sequenced, subcloned into 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 surface plasmon resonance in a BIAcore (Karlsson et al., 1991). 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). 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 (2.4×10^{-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 (2.4×10^{-9} M) compares favorably to the affinity of murine antibodies produced

against the same antigen 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 4 positions, 2 in H2, and 1 each in L1 and L3 (Table VI). Thus PM identified a subset of 'functional' residues whose mutation results in increased affinity. All but 1 of these 10 residues (V_λ L95) appear to have solvent accessible side chains in our C6.5 model. In contrast, two residues (V_λN30 and V_HY52) 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_H domain. In the V_λ domain, residues I28, G29, W91, and D92 are present in both C6.5 and KOL (Marquart et al., 1980), consistent with a structural role. The side chain of I28 is buried deep in the core of the V_λ domain between hydrophobic residues 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_λG29, V_λG95b, and V_HG53 are in turns and V_λW91 and V_λW96 pack against the V_H domain at the V_H-V_L interface. Hydrogen bonds between V_λD92 and V_λS27a and V_λN27b 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.

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 heavy or light chain shuffling C6.5 (2.4×10^{-9} M and 3.4×10^{-9} M

respectively) (Schier et al., in press). The 6 fold increase in affinity is also comparable to the 8 fold increase in affinity achieved using phage display and sequential mutagenesis of V_HCDR1 and V_HCDR3 of an anti-gp120 Fab (Barbas et al., 1994), or the 3 to 6 fold 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 subsets of residues: non-conserved functional residues which modulate affinity, and conserved residues, the majority of which have a structural role. To obtain even higher affinity antibodies, functional residues which 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 I.

Nucleotide mixtures for parsimonious mutagenesis of 19 amino acids located within V_λCDR1, V_λCDR3, and V_HCDR2 of C6.5 scFv[a].

V_λCDR1 >

AA[a]	I28[b]			G29			N30			N31			Y32		
nt[b]	N	N	K	N	N	T	N	N	K	N	N	K	N	N	K
T	3	91	90	5	5	100	3	3	90	3	3	90	91	3	90
C	3	3	0	5	5	0	3	3	0	3	3	0	3	3	0
A	91	3	0	5	5	0	91	91	0	91	91	0	3	91	0
G	3	3	10	85	85	0	3	3	10	3	3	10	3	3	10

V_λCDR3 >

AA	W91			D92			D93			S94			L95			S95a			G95b			W96		
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
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
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
A	5	5	0	3	91	0	3	91	0	5	7	0	5	5	0	5	7	0	5	5	0	5	5	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	5

V_HCDR2 >

AA	Y52			P52a			G53			D54			S55			D56		
nt	N	N	K	N	N	T	N	N	T	N	N	K	N	V	T	N	N	K
T	9	3	90	5	5	100	5	5	100	3	3	90	85	0	100	3	3	90
C	3	3	0	85	85	0	5	5	0	3	3	0	5	86	0	3	3	0
A	3	90	0	5	5	0	5	5	0	3	91	0	5	7	0	3	91	0
G	3	3	10	5	5	0	85	85	0	91	3	10	5	7	0	91	3	10

[a]**Methods:** PM-CAD was used for calculating nucleotide mixtures, as described in Balint & Larrick (1994), based on the most prevelant mutant having 5 non parental amino acids. Approximately 80% of the library should have 2 to 7 amino acid changes per scFv. Doping codons are according to IUB code [J. Biol. Chem. 261 (1986) 13] and nucleotide proportions are expressed as mole-percent.

[b]Single letter aa code is used, with the position number in the V_H or V_λ domain according to Kabat et al. (1987).

TABLE II.

Frequencies of parental and non-parental amino acids at C6.5 scFv residues subjected to parsimonious mutagenesis[a]

	V_λ CDR1>					V_λ CDR3>								V_H CDR2>					
	I28	G29	N30	N31	Y32	W91	D92	D93	S94	L95	S95a	G95b	W96	Y52	P52a	G53	D54	S55	D56
Glycine	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
Alanine	0.10	4.05	0.10	0.10	0.10		2.91	2.91	4.10		4.10	4.05		0.10	4.05	4.05	2.91	4.10	2.91
Proline	0.10	0.22	0.10	0.10	0.10		0.10	0.10	4.10		4.10	0.22		0.10	73.68	0.22	0.10	4.10	0.10
Valine	2.91	4.05	0.10	0.10	0.10	0.23	2.91	2.91		4.37		4.05	0.23	0.10	0.22	4.05	2.91		2.91
Leucine	3.19	0.22	0.11	0.11	0.38	3.94	0.11	0.11		73.68		0.22	3.94	0.38	4.05	0.22	0.11		0.11
Isoleucine	73.68	0.22	2.63	2.63	0.09	0.02	0.09	0.09		0.42		0.22	0.02	0.09	0.22	0.22	0.09		0.09
Methionine	7.89		0.28	0.28	0.01	0.21	0.01	0.01		3.95			0.21	0.01			0.01		0.01
Phenylalanine	2.63	0.22	0.09	0.09	2.63	0.42	0.09	0.09		7.90		0.22	0.42	2.63	0.22	0.22	0.09		0.09
Tryptophan	0.01		0.01	0.01	0.28	73.68	0.01	0.01		3.95			73.68	0.28			0.01		0.01
Serine	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
Cysteine	0.09	4.05	0.09	0.09	2.63	7.9	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
Threonine	2.91	0.22	2.91	2.91	0.10		0.10	0.10	4.10		4.10	0.22		0.10	4.05	0.22	0.10	4.10	0.10
Asparagine	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
Glutamine	0.02		0.56	0.56	8.17	3.94	0.56	0.56		3.95			3.94	8.18			0.56		0.56
Histidine	0.09	0.22	2.63	2.63	2.63		2.63	2.63	0.33		0.33	0.22		2.63	4.05	0.22	2.63	0.33	2.63
Tyrosine	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
Aspartate	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
Glutamate	0.01		0.28	0.28	0.28	0.21	7.90	7.90		0.21			0.21	0.28			7.90		7.90
Lysine	0.08		7.89	7.89	0.28	0.21	0.28	0.28		0.21			0.21	0.28			0.28		0.28
Arginine	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

[a]**Methods:** The frequency of parental and non parental aa at each position subjected to parsimonious mutagenesis was calculated from the nucleotide frequencies listed in Table I. The parental aa is listed across the top of each column, using single letter amino acid, with the position number in the V_H or V_λ domain according to Kabat et al. (1987). Amino acids are grouped into three categories, non-polar, polar, and charged.

TABLE III.

Sequences of primers used for construction of parsimonious mutagenesis phage antibody library[a]

LMB3	5′-CAGGAAACAGCTATGAC-3′
HuJλ2-3ForNot	5′-GAGTCATTCTCGACTTGCGGCCGCACCTAGGACGGTCAGCTTGGTCCC-3′
L1B	5′-ACCAAATACAGCCCGTCCTTCCAAGGCCAG-3′
L3B	5′-GTATCCTGGTACCAGCAGCTCCCAGGAAC-3′
L1F	5′-GAGTTTGGGGGCTGTTCCTGGGAGCTGCTGGTACCAGGATAC1,2,8,1,2,2,1,2,2,A,6,6,1,8,2,GTTGGA GCAGCTTCC-3′
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-3′
H2F	5′-GATGGTGACCTGGCCTTGGAAGGACGGGCTGTATTTGGT1,2,3,A,4,5,1,2,3,A,6,6,A,7,7,1,2,8,GATGA GCCCCATGACTC-3′

Nucleotide mixes

1. A(0.9), C(0.1); 2. T(0.91), C, A, G(0.03); 3. C(0.91), T, A, G (0.03); 4. G(0.86), T, C(0.07); 5. A(0.85), T, C, G(0.05); 6. C(0.85), T, A, G, (0.05); 7. G(0.85), T, C, 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)

[a]**Methods:** Oligos L1F, L3F, and H2F were synthesized by Keystone Laboratories, Palo Alto, CA and the remainder of the oligos were synthesized by Genset Inc, La Jolla, CA.

TABLE IV.

Frequency of binding scFv and percent of binding scFv with slower k_{off} than the parental scFv[a].

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

[a]**Methods:** Phage were subjected to 4 rounds of selection using decreasing concentrations of biotinylated c-erbB-2 extracellular domain (ECD). Phage were rescued, incubated with biotinylated c-erbB-2 ECD, captured with streptavidin coated M280 Dynabeads (Dynal), the beads washed, and the washed beads with bound phage used to infect *E. coli* TG1, exactly as described in Schier et al. (in press). Phage were prepared for the next round of selection, exactly as described in Schier et al. (in press).

[b]scFv was expressed (De Bellis & Schwartz, 1990) from 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. (1995). 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.

[c]scFv was expressed from 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 dialyzed periplasm using surface plasmon resonance in a BIAcore (Pharmacia) (Jønsson et al., 1991), exactly as described in Schier et al. (in press).

TABLE V.

Kinetics of selected scFv subjected to parsimonious mutagenesis[a]

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

[a]**Methods:** scFv DNA was subcloned into the vector pUC119Sfi1/Not1Hismyc exactly as described in Schier et al. (1995). The vector results in the addition of a hexahistidine tag at the C-terminus of the scFv. scFv was expressed (DeBellis & Schwartz), the periplasm harvested (Breitling et al., 1991) and dialyzed overnight against HBS. scFv was purified from the periplasmic preparation using immobilized metal affinity chromatography (Hochuli et al., 1988) and gel filtration, exactly as described in Schier et al. (1995). k_{on} and k_{off} were measured

on purified scFv using surface plasmon resonance in a BIAcore (Jønnson et al., 1991), as described in Schier et al. (in press). 1400 RU c-erbB-2 ECD (90 kDa) were coupled to a CM5 sensorchip (Johnsson et al., 1991) and k_{on} and k_{off} measured under continuous flow of 5 µl/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 100 nM c-erbB-2 ECD in the running buffer during the dissociation phase. K_d was calculated as k_{off}/k_{on}.

TABLE VI.

Deduced protein sequences of CDRs of C6.5 and C6.5 mutants from the parsimonious mutagenesis phage antibody library[a]

	V_HCDR2	V_λCDR1	V_λCDR3
Residue number[b]	5 5 5 6 0-2a3------0-----	2 2 2 3 4--7ab8-0----	8 9 9 9-----5ab6-
C6.5	LIYPGDSDIKYSPSFQG	SGSSSNIGNNYVS	AAWDDSLSGWV
3rd round selection			
PM1[c]	----------S--	-------MD----	-----T-----
PM2	F----------	--------K----	----E-WT---
PM3	S----NY----------	----------	----Y------
4th round selection			
PM4	----------	----------	----------
PM5	------YG--------	---------T--	----Y------
PM6	------YG--------	--------K----	----Y------
PM7	----------	----------	----H------
PM8	---A---A--------	----------	----Y------
PM9	----------	--------K----	------Y----
PM10	----------	--------D----	----A-QY---
PM11	----------	--------S----	-----A-----
PM12	----------	F----------	----E------
PM13	------YG--------	--------K----	----Y------
unselected clones			
1	----SN----------	-------F-----	----------
2	----------	-------S-----	--*E---C---
3	--F--VIE--------	---------N--	---Y-------
4	--*----N--------	----------	-----C-C---
5	-----EFN--------	----------	---EF------
6	----------	----------	-----A-TC--
7	---N-V----------	----------	--R----A---
8	----D-C---------	----------	-------A---

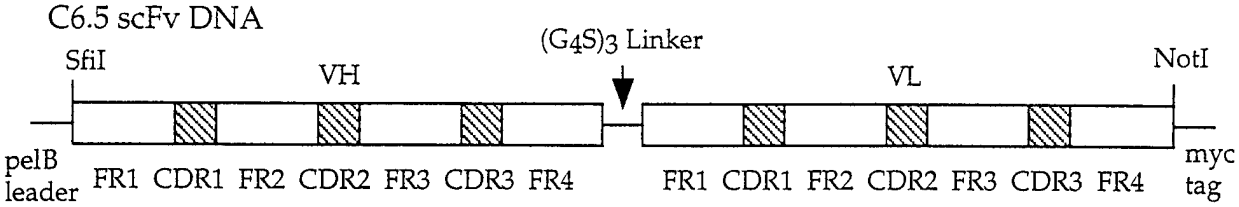
[a]**Methods:** The DNA of eight unselected scFv, and c-erbB-2 binding scFv from the third and fourth round of selection was sequenced (Sanger et al., 1977), the protein sequence deduced, and the sequences aligned with the deduced amino acid sequence of V_HCDR2, $V\lambda$CDR1, and $V\lambda$CDR3 of C6.5. Underlined residues are those subjected to parsimonious mutagenesis.

[b]Residue number is according to Kabat et al. (1987)

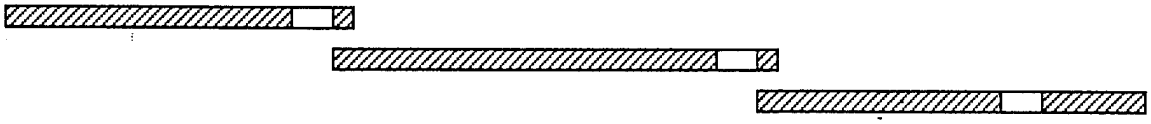
[c]Dashes indicate sequence homology with the original clone C6.5

Figure Legends

Figure 1. Construction of C6.5 scFv parsimonious mutagenesis phage antibody library. PCR was used to create an scFv gene repertoire where 19 amino acids located within CDR1 and CDR3 of the V_λ gene and CDR2 of the V_H gene were subjected to PM. Pairs of oligonucleotide primers (named in figure and 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) using PCR. Oligonucleotides H2F, L1F, and L3F encoded the 19 loop amino acids subjected to PM (white boxed area in gene segments). 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 7 times without primers (94°C for 30s, 60°C for 5s, 40°C for 5s (RAMP 5s) and 72°C for 60s) to join the fragments together. Primers were then added (LMB3 and L1F, 25 pmol each) and the mix was subjected to 30 cycles of amplification (94°C for 30s, 50°C for 30s and 72°C for 60s). This splicing and amplification process was 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_λ2-3ForNot to introduce a NotI restriction site at the 3′ end of the scFv gene repertoire. The resulting scFv gene repertoire was digested with SfiI and NotI, gel purified, and ligated into pCANTAB5E (Pharmacia) digested with SfiI and NotI. Aliquots of the ligation mixture were electroporated into electrocompetent *E. coli* TG1 (Gibson, 1984). FR, framework region; CDR, complementarity determining region; $(G_4S)_3$ linker encodes a 15 amino acid peptide linking the V_H domain to the V_λ domain.



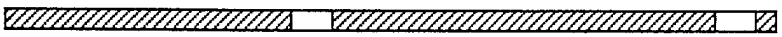
1) first round PCR



PM1 : 320 bp PM2 : 400 bp PM3 : 245 bp

primer : LMB3 / H2F L1B / L1F L3B / L3F

2) second round PCR



PM4 (PM1 + PM2) : 680 bp primer: LMB3 / L1F

3) third round PCR

PM5 (PM4 + PM3) : 880 bp primer: LMB3 / L3F

Appendix 4

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

Atty. Docket No. 02307E-061400/
UC Case No. 95-276-1

"Express Mail" Label No. TB383607983US

Date of Deposit June 15, 1995

I hereby certify that this is being deposited with the U.S. Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above, addressed to the
Asst. Commissioner of Patents and Trademarks,
Box Provisional Appln.
Washington, DC 20231.

By: Tom Hunter TH
Tom Hunter

BOX PROVISIONAL PATENT APPLICATION
ASST. COMMISSIONER OF PATENTS
AND TRADEMARKS
Washington, D. C. 20231

Sir:

Transmitted herewith for filing is a provisional patent application under 37 CFR 1.53(b)(2) of:

LAST NAME	FIRST NAME	MIDDLE INITIAL	RESIDENCE (CITY/STATE/COUNTRY)
Marks	James	D.	107 Ardmore Road, Kensington, CA 94707
Schier	Robert		1324 Willard Street, Apt. 301, San Francisco, CA 94117

Title: HIGH AFFINITY HUMAN ANTIBODIES TO NOVEL TUMOR ANTIGENS
Enclosed are:
[X] 97 pages of the specification.
[X] 4 sheet(s) of informal drawing(s).
[X] A verified statement to establish small entity status under 37 CFR 1.9 and 37 CFR 1.27.
[X] The invention was made by or under a contract with the following agencies of the United States Government: Army Contract No. DAMD17-94-J-4433 and the Department of Health and Human Services, National Institutes of Health, Grant No. ABBE CA51880.
[X] Declaration/unsigned
[X] Power of Attorney by Assignee and Exclusion of Inventor(s) under Rule 32/unsigned

Please charge Deposit Account No. 20-1430 as follows:
[X] Filing fee $ 75.00 (small)
[X] Any additional fees associated with this paper or during the pendency of this application.
2 extra copies of this sheet are enclosed.

Respectfully submitted,

TOWNSEND and TOWNSEND and CREW

Tom Hunter
Reg. No.: 38,498
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PROAPP.TRN 5/95

Applicant or Patentee: James D. Marks and Robert Schier Docket No. 02307E-061400
Serial or Patent No.: Unassigned U. C. Case No. 95-278-1
Filed or Issued: Herewith
For: HIGH AFFINITY HUMAN ANTIBODIES TO NOVEL TUMOR ANTIGENS

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS (37 CFR § 1.9(f) and § 1.27(d))--NONPROFIT ORGANIZATION

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

NAME OF ORGANIZATION: The Regents of the University of California
ADDRESS OF ORGANIZATION: 300 Lakeside Drive, 22nd Floor
Oakland, California 94612-3550

TYPE OF ORGANIZATION
[X] UNIVERSITY OR OTHER INSTITUTION OF HIGHER EDUCATION
[X] TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE [26 USC § 501(a) and § 601(c) (3)1]
[ ] NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OR THE UNITED STATES OF AMERICA
(NAME OF STATE _____)
(CITATION OF STATUTE _____)
[ ] WOULD QUALIFY AS TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE [26 USC § 501(a) and § 501(c) (3)] IF LOCATED IN THE UNITED STATES OF AMERICA
[ ] WOULD QUALIFY AS NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA IF LOCATED IN THE UNITED STATES OF AMERICA
(NAME OF STATE _____)
(CITATION OF STATUTE _____)

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 CFR § 1.9(e) for purposes of paying reduced fees under section 41(a) or (b) of Title 35, United States Code with regard to the invention entitled HIGH AFFINITY HUMAN ANTIBODIES TO NOVEL TUMOR ANTIGENS

by inventor(s) James D. Marks and Robert Schier described in

[X] the specification filed herewith
[ ] application serial no. _____, filed _____
[ ] Patent no. _____, issued _____

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above identified invention [except for a license to a Federal Agency pursuant to USC § 202 (c) (4)].

If the rights held by the nonprofit organization are not exclusive, each individual, concern, or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who would not qualify as an independent inventor under 37 CFR § 1.9(c) if that person made the invention, or by any concern which would not qualify as a small business concern under 37 CFR § 1.9(d) or a nonprofit organization under 37 CFR § 1.9(e).
*NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities (37 CFR § 1.27).

NAME N/A
ADDRESS
[ ] INDIVIDUAL [ ] SMALL BUSINESS CONCERN [ ] NONPROFIT ORGANIZATION

NAME
ADDRESS
[ ] INDIVIDUAL [ ] SMALL BUSINESS CONCERN [ ] NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. [37 CFR § 1.28(b)]

I hereby declare that all statements made 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 are punishable by fine or imprisonment, or both, under 18 USC § 1001, and may jeopardize the validity of the application, any patent issuing thereon, or any patent which this verified statement is directed.

NAME OF PERSON SIGNING: David J. Aston
TITLE IN ORGANIZATION: Assistant Director, Office of Technology Transfer
ADDRESS OF PERSON SIGNING: 300 Lakeside Drive, 22nd Floor
Oakland, California 94612-3550

SIGNATURE _____ DATE June 14, 1995

Attorney Docket No.: 02307E-061400
UC Case No.: 95-276-1

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of)
)
James D. Marks *et al.*)
)
Serial No.:)
)
Filed: herewith)
)
For: HIGH AFFINITY HUMAN)
ANTIBODIES TO NOVEL)
TUMOR ANTIGENS)
)

POWER OF ATTORNEY BY ASSIGNEE
AND EXCLUSION OF INVENTOR(S) UNDER RULE 32

Hon. Assistant Commissioner of Patents
and Trademarks
Washington, D.C. 20231

Sir:

The undersigned assignee of the entire interest in the above-identified subject application hereby appoints Albert J. Hillman, Reg. No. 20,134; William M. Smith, Reg. No. 30,223; and Kenneth A. Weber, Reg. No. 31,677; Kevin L. Bastian, Reg. No. 34,774; Ellen Lauver Weber, Reg. No. 32,762; Tom Hunter, Reg. No. 38,498; all of the firm of Townsend and Townsend Khourie and Crew, as its attorneys to prosecute this application and to transact all business in the Patent Office connected therewith, said appointment to be to the exclusion of the inventors and their attorney(s) in accordance with the provisions of Rule 32 of the Patent Office Rules of Practice.

Please direct all telephone calls to Tom Hunter at (415) 543-9600 and all correspondence relative to said application to the following address:

TOWNSEND and TOWNSEND KHOURIE and CREW
Steuart Street Tower, 20th Floor
One Market Plaza
San Francisco, California 94105

ASSIGNEE: THE REGENTS OF THE UNIVERSITY OF CALIFORNIA

Signature: _____

Typed Name:

Title:

Address: 300 Lakeside Drive, 22nd Floor
Oakland, California 94612-3550

Dated: _____

Attorney Docket No. 02307E-061400
UC Case No. 95-276-1

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: **HIGH AFFINITY HUMAN ANTIBODIES TO NOVEL 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 applications(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

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.**	
Residence & Citizenship	City **Kensington**	State/Foreign Country **California**	Country of Citizenship **U.S.A.**	
Post Office Address	Post Office Address **107 Ardmore Road**	City **Kensington**	State/Country **California**	Zip Code **94707**
Full Name of Inventor 2	Last Name **Schier**	First Name **Robert**	Middle Name or Initial	
Residence & Citizenship	City **San Francisco**	State/Foreign Country **California**	Country of Citizenship **U.S.A.**	
Post Office Address	Post Office Address **1324 Willard Street, Apt. 301**	City **San Francisco**	State/Country **California**	Zip Code **94117**
Full Name of Inventor 3	Last Name	First Name	Middle Name or Initial	
Residence & Citizenship	City	State/Foreign Country	Country of Citizenship	
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

DEC.MRG 5/95

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Attorney Docket No. 2307E-614
Client: 95-276-1

PROVISIONAL PATENT APPLICATION

NOVEL HIGH AFFINITY HUMAN ANTIBODIES TO TUMOR ANTIGENS

Inventors:

James D. Marks, a citizen of the United States of America

Robert Schier, a citizen of Austria

Assignee:

The Regents of the University of California

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PATENT
Attorney Docket No. 2307E-614
Client Ref: 95-276-1

NOVE HIGH AFFINITY HUMAN ANTIBODIES TO TUMOR ANTIGENS

BACKGROUND OF THE INVENTION

This application is related to a provisional application filed on June 14, 1995.

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.

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.

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.*).

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 $(Fab')_2$, and the dimer $(sFv')_2$.

In a particularly preferred embodiment, the C6 antibody has a K_d ranging from about 1.6×10^{-8} to about 1×10^{-12} 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.

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 Examples 1, 2 or 3.

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 procedes 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 procedes 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

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 F(ab)'$_2$, a dimer of Fab which itself is a light chain joined to V_H-C_H1 by a disulfide bond. The F(ab)'$_2$ may be reduced under mild conditions to break the disulfide linkage in the hinge region thereby converting the (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 pepetide linker) to form a continuous polypeptide.

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 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 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).

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 Kd 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

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 1.6×10^{-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 Cα-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 ß-strand which forms part of the V_H-V_L interface. C6H2 has 2 conservative mutations located in the ß-strand formed by framework 3 residues.

Figure 3. Locations of mutations in light chain shuffled sFv which spontaneously form dimers. Mutations are indicated as shaded spheres on the α-carbon tracing of the Fv fragment of the immunoglobulin KOL (Marquart et al., 1980) with the V_L 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 ß-strand which forms part of the V_H-V_L interface.

DETAILED DESCRIPTION

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 elicits a little or no immungenic 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 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, 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 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 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 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, FcγI, FcγII and FcγIII receptors) on the 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 response. Thus the C6 antibody receptor may be conjugated to a drug such as vinblastine, vindesine, melphalan, N-Acetylmelphalan, methotrexate, aminopterin, doxirubicin, 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.

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 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.

5

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 10 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.

15 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 20 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* 25 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); 30 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.

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), Qβ-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, I. *et al.*, 1983, *Gene* 22:229-235; Mosbach, K. *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 1μM or better, with preferred embodiments having a K_D of 1 nM or better and most preferred embodiments having a K_D of 0.1nM 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 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 minutes. The bacteria are then pelleted by centrifugation at 6000g 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 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).

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 .

Modification of C6 Antibodies

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 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 (38, 39). For example, a sFv gene coding for the V_H and V_L domains of an anti-lysozyme antibody (Dl.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 (38).

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 (38). 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 (38). Thus, even when enrichments in each round are low (33),

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 (33).

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 (39). 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 (40, 41), *e.g.,* followed by incubation with polyclonal anti-mouse Fc conjugated to horseradish peroxidase.

Phage display can be used to increase antibody affinity

To create higher affinity antibodies, mutant sFv gene repertories, 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_H or V_L gene from a binding sFv with a repertoire of nonimmune V_H or V_L genes (chain shuffling) (45). Such gene repertoires contain numerous variable genes derived from the same germline gene as the binding sFv, but with point mutations (34). Using light chain shuffling and phage display, the binding avidities of a human sFv antibody fragment can be dramatiaclly 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) (34). Shuffling

of the V_H gene, while leaving the V_H CDR3 and new light chain intact, further improved the affinity from 15 nM to 1 nM.

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-erb-B2 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-erb-B2 expressed on cells, and thus this sFv was selected for further characterization.

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.

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. k_{on} is typically measured in the range 1.0×10^{2} to 5.0×10^{6} and k_{off} in the range 1.0×10^{-1} to 1.0×10^{-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.

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 1.6×10^{-8} M determined by BIAcore is in close agreement to the K_d determined by Scatchard analysis after radioiodination (2.0×10^{-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-erB-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-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 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.

	741F8	C6.5
K_d (BIAcore)	$2.6x10^{-8}$ M	$1.6x10^{-8}$ M
K_d (Scatchard)	$5.4x10^{-8}$ M	$2.1x10^{-8}$ M
K_{on} (BIAcore)	$2.4x10^{5}$ $M^{-1}s^{-1}$	$4.0x10^{5}$ M^{-1} s^{-1}
K_{off} (BIAcore)	$6.4x10^{-3}$ s^{-1}	$6.3x10^{-3}$ 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	5.6a
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.

Estimating the affinity of unpurified sFv for c-erbB-2

Phage display and selection generally results in the selection of higher affinity mutant sFvs (34-36, 45), but probably does not result in the separation of mutants with less than a 6 fold difference in affinity (36). 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_{off}, measurement of k_{off} should identify higher affinity sFv. k_{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_{off} is independent of concentration. The value of k_{off} for periplasmic and purified sFv is in close agreement (Table 2).

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

sFv	K_{off} (s^{-1})
C6-5 periplasm	5.7×10^{-3}
C6-5 purified	6.3×10^{-3}
C6-5ala3 periplasm	9.3×10^{-3}
C6-5ala3 purified	1.5×10^{-3}
C6-5ala10 periplasm	3.7×10^{-3}
C6-5ala10 purified	4.1×10^{-3}

Ranking of sFv by k_{off}, and hence relative affinity, can be determined without purification. Determination of relative affinity without purificaiton 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).

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 (39) and after transformation a library of 2×10^5 transformants was obtained. Phage were prepared and concentrated as described in Example 1 or 2.

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 (35). After four rounds of selection, 62/90 clones analyzed produced sFv which bound c-erbB-2 by ELISA. sFv 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. sFvs 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 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.

sFv clone	K_d (M)	K_{on} (M^{-1} s^{-1})	K_{off} (s^{-1})
C6.5	1.6×10^{-8}	4.0×10^5	6.3×10^{-3}
C6L1 (light chain shuffle)	2.6×10^{-9}	7.8×10^5	2.0×10^{-3}
C6VHB-4 (heavy chain shuffle)	4.8×10^{-9}	1.25×10^6	6.0×10^{-3}
C6VHC (heavy chain shuffle)	3.1×10^{-9}	8.4×10^5	2.6×10^{-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 1×10^6 transformants. Selections were performed on biotinylated c-erbB-2 and 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 BlAcore (Table 3). The affinity of C6.5 was increased 5 fold by heavy chain shuffling and 6 fold by light chain shuffling.

Increasing the affinity of C6-5 by site directed mutagenesis of the third CDR of the light chain

The majority of antigen contacting amino acid side chains are located in the complementarity determining regions (CDRs), three in the V_H (CDRl, CDR2, and CDR3) and three in the V_L (CDRl, CDR2, and CDR3) (58-60). 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 (56, 57). Thus mutation (randomization) of the CDRs and screening against c-erbB-2 may be used to generate C6 antibodies having improved binding affinity. For example, to increase the affinity of C6.5 for c-erbB-2, 7 amino acids in V_L CDR3 were partially randomized by synthesizing a 'doped' oligonucleotide in which the wild type nucleotide occurred with a frequency of 70%, and the other three nucleotides at a frequency of 10%. 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 1×10^7 transformants.

Selection of the C6.5 mutant VL CDR3 library 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. sFv was expressed from 24 ELISA positive clones from the 3rd and 4th rounds of selection, the periplasm harvested, and the k_{off} determined by BlAcore. 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. sFv was expressed, purified by IMAC and gel filtration, and sFv affinity and binding kinetics determined by BlAcore (Table 4). Mutant sFv were identified with 16 fold increased affinity for c-erbB-2.

Table 4. Amino acid sequence, affinity, and kinetics of binding of C6.5 light chain CDR3 mutants. Affinity and kinetics of binding were determined on purified, gel filtered sFv in a BIAcore. A hyphen "-" indicates there 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	AAWDDSLSGWV	1.6×10^{-8}	4.0×10^{5}	6.3×10^{-3}
3rd Round of selection				
C6ML3-5	----Y------	3.2×10^{-9}	5.9×10^{5}	1.9×10^{-3}
C6ML3-2	----H------	2.8×10^{-9}	7.1×10^{5}	2.0×10^{-3}
C6ML3-6	-S--Y------	3.2×10^{-9}	5.9×10^{5}	1.9×10^{-3}
C6ML3-1	----Y--W---	6.7×10^{-9}	3.0×10^{5}	2.0×10^{-3}
C6ML3-3	-T--YA-----	4.3×10^{-9}	4.6×10^{5}	2.0×10^{-3}
C6ML3-7	----YAV----	2.6×10^{-9}	6.5×10^{5}	1.7×10^{-3}
C6ML3-4	-S-EY--W---	3.5×10^{-9}	4.0×10^{5}	1.4×10^{-3}
4th Round of selection				
C6ML3-12	----Y-R----	1.6×10^{-9}	4.5×10^{5}	7.2×10^{-4}
C6ML3-9	-S--YT-----	1.0×10^{-9}	6.1×10^{5}	9.2×10^{-4}
C6ML3-10	---E-PWY---	2.3×10^{-9}	6.1×10^{5}	1.4×10^{-3}
C6ML3-11	----YA-W---	3.6×10^{-9}	6.1×10^{5}	2.2×10^{-3}
C6ML3-13	----AT-W---	2.4×10^{-9}	8.7×10^{5}	2.1×10^{-3}
C6ML3-8	----HLRW---	2.6×10^{-9}	6.5×10^{5}	1.7×10^{-3}
C6ML3-14	-----P-W---	1.0×10^{-9}	7.7×10^{5}	7.7×10^{-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.

Creation of C6.5 (sFv')2 homodimers and effects on affinity and binding kinetics for cerbB-2

To create C6 $(sFv')_2$ antibodies, two C6 sFvs are joined, either through a linker or through a disulfide bond betwee, for example, two cysteins. 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 $(sFv')_2$ 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 sFvs were incubated together to form $(sFv')_2$ and the resulting material analyzed by gel filtration. 50% of the monomer was converted to $(sFv')_2$ homodimer as determined by gel filtration and nonreducing polyacrylamide gel electrophoresis. The affinity of the C6.5 sFv' monomer and (sFv')2 dimer were determined by BIAcore (Table 5). The apparent affinity (avidity) of C6.5 increases 40 fold when converted to an $(sFv')_2$ homodimer.

Table 5. Affinities and binding kinetics of C6.5 sFv and C6.5 $(sFv')_2$.

Clone	K_d (M)	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})
C6.5 monomer	1.6×10^{-8}	4.0×10^{5}	6.3×10^{-3}
C6.5 dimer	4.0×10^{-10}	6.7×10^{5}	2.7×10^{-4}

C6.5 $(sFv')_2$ exhibits a significant avidity effect compared to the sFv. Thus, this approach increases antibody fragment affinity, while remaining below the renal threshold for excretion.

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_d ranging from 1.0×10^{-6} M to 1.0×10^{-9} 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_{off} 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.

In vivo binding and biodistribution results showed that the overall tumor targeting was lower than usually observed, as evidenced by 24 hour retention of only 0.4% ID/g in tumors for ^{125}I-C6.5, which usually exhibits two-fold higher tumor retention (Table 1). This experiment demonstrates a clear contribution of affinity to overall tumor retention and targeting specificity, with tumor retention of 0.14 %ID/g for ^{125}I-C6.Gly4Ala (K_d= 1 x 10^{-7} M), and 0.78 %ID/g for ^{125}I-C6L1 (K_d=2.5 x 10^{-9} M; p = 0.00056). In a confirmatory study, the 24 hour tumor retention of C6.5 was 0.67 %ID/g, while that of C6L1 was 1.13 %ID/g (p = 0.048). C6ML3-4, a variant with binding characteristics similar to those reported above for C6L1, showed equivalent tumor retention in the same assay.

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.

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 x 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 x 10^{-9} M). To simplify affinity measurement, C6ML3-9, or other lower affinity C6 antibodies, are used as a template, rather than a higher affinity sFv. The CDR sequences of the highest affinity mutants from each CDR

library are combined to obtain an additive increase in affinity. A similar approach has been used to increase the affinity of human growth hormone (hGH) for the growth hormone receptor over 1500 fold from 3.4 x 10-10 to 9.0 x 10-13 M (56).

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 (61). 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 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 C6NIL3-Bl 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.

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. sFv from twenty to forty ELISA positive clones are expressed in 10 ml cultures, the 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. sFv 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 1.6×10^{-10} M (see Example 3).

In vitro cell binding assays, in vivo pharmacokinetic and biodistribution studies

Once higher affinity sFv 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. sFv 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 the enclosed

manuscript. Since the $t_{1/2}$ of retention should be at least 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 BlAcore 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 therepeutics *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 1.6×10^{-8} M and 1.0×10^{-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 1.0×10^{-11} is 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 >20 hours. 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 1.6×10^{-8} M to 1.0×10^{-10} M are provided. Combining these mutations into the same sFv produces a mutant sFv with a K_d between 1.6×10^{-10}M and 3.3×10^{-11} M.

Preparation of C6 (sFv)2, Fab, and (Fab')2 molecules

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 $(sFv')_2$ is created from the parent sFv as described above and in Example 1. An sFv gene can be excised, *e.g.*, with NcoI and NotI from pHEN-1 or pUC119 Sfi-NotmycHis and cloned into pUC119/C6.5mycCysMHIS, 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 cystine 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')2 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')2, which is purified. This approach was used to produce C6.5 (SFv')2 dimer, which demonstrates a 40 fold higher affinity than C6.5. A (SFv')2 may be constructed for example, from C6L1 (K_d = 2.5 x 10^{-9} M) and C6ML3-9 (K_d = 1.0 x 10^{-9} M). As higher affinity SFv become available, their genes are similarly used to construct $(SFv')_2$.

C6.5 based Fab are expressed in *E. coli* using an expression vector similar to the one described by Better *et. al.* (26). 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_H gene. The vector also contains the lac promoter, a pelb leader sequence to direct expressed V_H-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 V_L gene is spliced to the C_L gene using PCR and cloned into the vector containing the V_H CH1 gene.

<u>Preparation of Chimeric Molecules</u>

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.

Cytotoxins

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

***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).

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).

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).

The targeting molecule can be inserted in replacement for domain Ia. A similar insertion has been accomplished in what is known as the TGFα-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).

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).

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.

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.

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.*, ^{3}H, ^{125}I, ^{35}S, ^{14}C, or ^{32}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.

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, TGFα, and the like.

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, doxirubicin, 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).

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.

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 ($-NH_2$) 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 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 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.

Production of Fusion Proteins

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 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.

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.*, 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.

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); 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

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.

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 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 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.

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, 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.

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.

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 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.

5

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.

10 *In Vivo* Detection of c-erB-2

The 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 oranism a chimeric molecule comprising a C6 joined to a label detectable *in vivo*. Such labels are well known to those of skill in 15 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 20 detected and localized using the appropriate detection method.

In Vitro Detection of c-erB-2 peptides

The C6 antibodies of this invention are useful for the detection of c-erbB-2 *in vitro e.g.*, in biological samples obtained from an organism. The detection and/or 25 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. 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 30 indicative of cells overexpressing c-erbB-2. Particularly preferred biological samples include blood, urine, and tissue biopsies.

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. Although the sample is typically taken from a human patient, the assays can be used to detect c-erbB-2 antigen in samples from any mammal, such as dogs, cats, sheep, cattle, and pigs.

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.

Quantification of c-erbB-2.

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

Immunological Binding Assays

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-erB-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-erB-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-erB-2 peptide, the anti-c-erB-2 antibody/c-erB-2 peptide complex, or to a modified capture group (*e.g.*, biotin) which is covalently linked to c-erB-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.

(i) 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-erb-2 peptide.

(ii) Competitive Assay Formats

In competitive assays, the amount of analyte (*e.g.*, c-erB-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-erb-2 is added to a test sample with an unquantified amount of c-erB-2, and the sample is contacted with a capture agent, *e.g.*, a C6 antibody that specifically binds c-erb-2. The amount of added c-erB-2 which binds to the C6 antibody is inversely proportional to the concentration of c-erB-2 present in the test sample.

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

Assays for erb-2 and C6 antibody

A. *Sample Collection and Processing*

An antibody or polypeptide is preferably quantified in a biological sample, such as a cell, or a tissue sample derived from a patient. In a preferred embodiment, erB-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 erB-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.

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.

B. *Quantification of antibodies and polypeptides.*

C6 antibodies and c-erB-2 polypeptides are detected and quantified by any of a number of 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, immunodiffusion (single or double), immunoelectrophoresis, radioimmunoassays (RIAs), enzyme-linked immunosorbent assays (ELISAs), immunofluorescent assays, and the like.

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-erB-2, C6 antibody, or other capture agent immobilized on a solid substrate, it is 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.

D. *Other Assay Formats*

Western blot analysis can also be used to detect and quantify the presence of erB-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 erB-2 peptide or the anti-erB-2 antibody. The antibodies specifically bind to the biological agent of interest on 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-erB-2 or erB-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), which is incorporated herein by reference.

E. *Labels*

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.*, ^{3}H, ^{125}I, ^{35}S, ^{14}C, or ^{32}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. 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, 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.

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 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,

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-erB-2 peptides. In this case, antigen-coated particles are agglutinated by 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.

F. *Substrates*

As mentioned above, depending upon the assay, various components, including the erB-2, C6 or antibodies to erB-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 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 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.

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).

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.

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.

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.

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 FcγI, FcγII and FcγIII, 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.

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 sFvs binding c-erbB-2

Materials and Methods:

Preparation of c-erbB-2 ECD

The antigen c-erbB-2 ECD with a Ser-Gly-His_6 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 (3×10^7 members) expressing sFv as pIII fusions on the phage surface (Marks, J.D. 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 μg/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, 7.0×10^{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 μg/ml ampicillin and 2.5 μg/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 NaH_2PO_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, J.D. et al., (1991) J. Mol. Biol. 222:581-597). Briefly, immunotubes (Nunc, Maxisorb) were coated with 2 ml (100 μg/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, P. 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 μg/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, P. et al., (1985) Nucleic Adds Res., 13: 4431-43) (A_{600} run ~ 0.8) were infected with 100 μl 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 (Hoogenboom, H.R. et al., (1991) Nucleic Acids Res. 19:4133-4137). Single ampicillin resistant colonies were used to inoculate microtire plate wells containing 150 μl of 2 x TY containing 100 μg/ml ampicillin and 0.1% glucose. The bacteria were grown to an A_{600} nm ~ 1.0, and sFv expression induced by the 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 μg/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 hours at 20°C with 50 μl 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 (Munro, S. et al., (1986) Cell, 46:291-300) and peroxidase conjugated anti-mouse Fc antibody (Sigma) using ABTS as substrate (Ward, E. S. et al., (1989) Nature, 341:544-546). The 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 μg/ml of bovine serum albumin, hen egg white lysozyme, bovine glutamyltranspeptidase, c-erbB-2 ECD, VCS M13 (3.5×10^{12}/ml) and casein (0.5%). For ELISA with biotinylated c-erbB-2 ECD, microtiter plates (Immunolon 4, 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 1 h at 37°C and incubated with 50 μl biotinylated c-erbB-2 extracellular domain (5 μg/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).

Subcloning, expression and purification.

To facilitate purification, the C6.5 sFv gene was subcloned into the expression vector pUC119Sfi/NotHismyc (Griffiths, A.D. 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 pUC119Sfil/NotIHismyc digested with NcoI and NotI and the legation 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 pUC119Sfil/NotIHismyc. The culture was grown at 37°C to an A_{600} 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 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.

Affinity and kinetic measurements

The K_d of C6.5 and 74IF8 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 μg/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 μl/min. k_{on} was determined from a plot of (1n (dR/dt))/t vs concentration (Karlsson, R., et al., (1991) J. Immunol. Methods., 145:229-240). K_{off} was determined from the dissociation part of the sensorgram at the highest concentration of sFv analyzed (Johnsson, B. et al., (1991) Anal. Biochem., 198:268-277). The K_d of C6.5 was also determined by Scatchard analysis (Scatchard, G.

(1949) Annals N.Y. Acad. Sci., 51:660). All assays were performed in triplicate. Briefly, 50 μg of radioiodinated sFv was added to 5 x 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

The C6.5 sFv was labeled with radioiodine using the CT method (DeNardo, S. J. 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 μg of CT (Sigma, St. Louis, MO) was added per 100 μg of protein and the resulting mixture was incubated for three minutes at room temperature. The reaction was quenched by the addition of 10 μg of sodium metabisulfite (Sigma) per 100 μg of protein. Unincorporated radioiodine was separated from the labeled protein by gel filtration using the G-50-80 centrifuged-column method (Adams, G.P. et al., (1993) Cancer Res. 53:4026-4034). The final specific activity of the CT labelling was 1.4 mCi/mg for the ^{131}I-C6.5 sFv and typically about 1.0 mCi/mg for the ^{125}I-C6.5 sFv.

Quality Control

The quality of the radiopharmaceuticals was evaluated by HPLC, SDS-PAGE, and a live cell binding assay as previously described (Adams, G.P. 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 ^{125}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) (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, G.P. et al., (1993) Cancer Res. 53:4026-4034).

Cell Surface Dissociation Studies

Cell surface retention of biotinylated forms of the sFv molecules were measured by incubating 2 μg of either sFv with 2 x 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 300g, the supenatants 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 to retention at 0 min at 4°C.

Biodistribution and Radioimmunoimaging Studies

Four to six week old C.BI7/Icr-*scid* mice were obtained from the Fox Chase Cancer Center Laboratory Animal Facility. 2.5 x 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}I-C6.5 sFv was diluted in PBS to a concentration of 0.2 mg/ml and each mouse was given 100 μL, containing 20 μg 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, G.P. et al., (1993) Cancer Res. 53:4026-4034; Adams, G.P. et al., (1992) Antibody Immunoconj. 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 μg (100 μl) of ^{131}I-C6.5. At 24 hours after injection, the mice were euthanized by asphyxiation with CO_2 and images were acquired on a Prism 2000XP gamma camera (Picker, Highland Heights, OH 44142). Preset acquisitions of 100k counts were used.

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_λ 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

Table 6

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
C6.1	QVQLVESGGGLVQPGGSLRLSCAASGFTFS	SYEMN	WVRQAPGKGLEWVS	YISSSGSTIYYADSVKG	RFTISRDNAKNSLYLQMNSLRAEDTAVYYCAR	DLGGYSYGYVGLDY	WGQGTLVTVSS
DP58	E----------	-----	----------	----------	----------		
C6.5	QVQLLQSGAELAKPGESLRISCKGSGYSFT	SYWIA	WVRQMPGKGLEYMG	LIYPGDSDTRYSPSFQG	QVTISVDKSVSTAYLQWSSLKPSDSAVYFCAR	HDVGYCSSSSCAKWPEYFQH	WGQGTLVTVSS
DP73	E---V-----V----------	----Q	----------M--	I---------R-------	-----A---I----------A--T-M-V---		

Light chains

	Framework 1	CDR1	Framework 2	CDR2	Framework 3	CDR3	Framework 4
C6.1	SELTQDPAVSVALGQTVRITC	QGDSLRSYYAS	WYQQKPGQAPVLVIY	GKNNRPS	GIPDRFSGSSSGNTASLTITGAQAEDEADYYC	NSRDSSGNHVAV	FGGGTKVTVLG
IGLV3S1	-S----------	----------	----------	-------	----------T----------	--------N V-	
C6.5	QSVLTQPPSVSAAPGQKVTISC	SGSSSNIGRNYVS	WYQQLPGTAPKLLIY	GHTNRPA	GVPDRFSGSKSGTSASLAISGPASEDEADYYC	AAWDDSLSG HV	FGGGTKLTVLG
HUMLV122	----------	----------	----------	DNNK--S	-S----------T-Q-T-LQTG-------	GE--S---A	
DPL5	----------A-GT---R-----	--------S---Y	----------	RNNQ--S	----------L----------	----------	

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 (1.6×10^{-8} M) agreed closely to the value determined by Scatchard (2.0×10^{-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}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.

	741F8	C6.5
K_d (BIAcore)	$2.6x10^{-8}M$	$1.6x10^{-8}M$
K_d (Scatchard)	$5.4x10^{-8}M$	$2.1x10^{-8}M$
K_{on} (BIAcore)	$2.4x10^{5}M^{-1}s^{-1}$	$4.0x10^{5}M^{-1}s^{-1}$
K_{off} (BIAcore)	$6.4x10^{-3}s^{-1}$	$6.3x10^{-3}s^{-1}$
% 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

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_H gene 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 binding scFv as a BssHII-NotI fragment. To create the libraries three V_H gene repertoires enriched for human V_H1, V_H3, and V_H5 gene were amplified by PCR using as a template single stranded DNA prepared from a 1.8×10^8 member scFv phage antibody library pHEN-1 (Marks *et al.* (1991) *J. Mol. Biol.* 222, 581-597). For PCR, 50 ul reactions were prepared containing 10 ng template, 25 pmol back primer (LMB3), 25 pmol forward primer (PV_H1FOR1, PV_H3FOR1, or PV_H5FOR1), 250 uM-dNTPs, 1 mM MgCl2, and 0.5 ul (2 units) Taq DNA polymerase (Promega) in the manufacturer's buffer. Primers PV_H1For1, PV_H3For1, and PV_H5For1 were designed to anneal to the consensus V_H1, V_H3, or, V_H5 3' FR3 sequence respectively (Tomlinson *et al.* (1992) *J. Mol. Biol.* 227, 776-798; see Table 14). The reaction mixture was subjected to 25 cycles of amplification (94°C for 30 sec, 55 °C for 30 sec and 72°C for 30sec) 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 ul of water (Sambrook *et al.* (1990)).

The DNA fragments from the first PCR were used as templates for a 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 performed as described above using 200 ng purified first PCR product as template and the back primers PV_H1For2, PV_H3For2, and PV_H5For2. The PCR products were purified by extraction with phenol/chloroform, precipitated with ethanol, resuspended in

50 ul water and 5 ug 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.* 1991a *supra*) digested with NotI and NcoI. The ligation mix was purified by extraction with phenol/chloroform, ethanol precipitated, resuspended in 20 ul water, and 2.5 ul samples electroporated (Dower *et al.* (1988) *Nucleic Acids Res.* 16, 6127-6145) into 50 ul *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 3min and then plated on TYE (Miller (1972) *Experiments in Molecular Genetics* Cold Springs Harbor Lab Press, Cold Springs Harbor, New York) media containing 100 ug 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 ug ampicillin/ml, 1% glucose (2 x 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 (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_H1rep, pHEN-1-V_H3rep and pHEN-1-V_H5rep.

Three separate C6.5 heavy chain shuffled phage antibody libraries were made from the pHEN-1-V_H1rep, pHEN-1-V_H3rep, and pHEN-1-V_H5rep 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 intpHEN-1-V_H1rep, pHEN-1-V_H3rep, and pHEN-1-V_H5rep 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 ipHEN-1 containing human V_k and V_λ 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_λ gene repertoire was amplified by PCR from a 1.8×10^8 member scFv 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_H1, 2, 6, J_H3, or J_H 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_λ3S1 (Hoogenboom and Winter (1992) *J. Mol. Biol.* 227, 381-388) digested with XhoI and NotI. Transformation of *E. coli,* TG1, PCR screening, and creation of library stocks was as described above. The resulting phage library was termed pHEN-1-$V_L$rep.

The light chain shuffled phage antibody library was made for pHEN-1-$V_L$rep. The C6.5 V_H gene was amplified by PCR from pHEN-1-C6.5 plasmid DNA using the primers PC6V_H1For and LMB3. The PCR reaction mixture was purified, digested with XhoI and NcoI, gel purified and ligated into pHEN-1-$V_L$rep digested with Xho and NcoI. Transformation of *E. coli* TG1, PCR screening, and creation of library stocks was as described above.

Construction of sFv containing highest affinity V_H and V_L gene obtained by chain shuffling

Two new scFv were made by combining the V_L gene of the highest affinity light chain shuffled scFv (C6L1) with the V_H gene of the highest affinity heavy chain shuffled scFv (C6H1 or C6H2). The C6L1 plasmid was digested with NcoI and XhoI to remove the C6.5 V_H gene and gel purified. The V_H gene of C6H1 or C6H2 was amplified by PCR using the primers LMB3 and PC6V_H1For, 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.

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 ul) from the library stocks to give an A_{600} of 0.3 to 0.5 and grown for 30 min, shaking at 37°C. About 1 x 10^{12} 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 ug ampicillin/ml and 50 ug

kanamycin/ml (2 x TY-AMP-KAN), and grown overnight, 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 (25 mM NaH2PO4, 125 mM NaCl, pH 7.0, PBS) and filtered through a 0.45 u filter. The phage preparation consistently resulted in a titre of approximately 10^{13} transducing units/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 ECD (25 ug/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^{12}/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.

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^{13} 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.

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 ul (round 1), 100 ul (round 2) or 50 ul (rounds 3 and 4) of beads were incubated with the phage-antigen mixture for 15 min, rotating on an under-and-over-turntable at room temperature. In S2, 100 ul (round 1) or 50 ul (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 ul 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 tc-erbB-2 was performed by ELISA using bacterial supernatant containing expressed scFv. Expression of scFv (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 ul 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 scFv in the bacterial supernatant. For selection performed on Immunotubes, ELISA plates (Falcon 3912) were incubated with c-erbB-ECD (2.5 ug/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 ug/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 scFv to c-erbB-2 ECD was detected with the mouse monoclonal antibody 9E10 (1 ug/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_H and V_L genes (Sanger *et al.* (1977) *Proc Natl Acad Sci U. S. A.* 74, 5463-5467). Sequence data has been deposited with the GenBank Data Library, accession numbers (pending).

Screening of scFv 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 ug/ml) at 4°C overnight. Biotinylated c-erbB-2 ECD (5 ug/ml) was added to the wells and incubated for 30 min at room temperature. Bacterial supernatant containing scFv was incubated with varying concentrations of c-erbB-2 (0 to 100 nM) at 4°C for 1 h. The amount of free scFv was then determined by transferring 100 ul of each mixture into the wells of the previously prepared ELISA plate and incubating for 1h at 4°C. Binding of scFv was detected as under ELISA screening and the IC50 calculated as described (Friguet *et al.* (1985), *supra*)

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). 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 induced with IPTG (De Bellis and Schwartz, 1990). Cultures were grown overnight at 25°C, scFv 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, HBS). In a BIAcore flow cell, approximately 1400 resonance units (RU) of c-erbB-ECD (25 ug/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 scFv in the periplasmic fraction was measured under a constant flow of 5 ul/min. An apparent dissociation rate constant (k_{off}) was determined from the dissociation part of the sensorgram for each scFv 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 $MgCl_2$ 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 scF

To facilitate purification, shuffled scFv genes were subcloned (Example 1) into the expression vector pUC11Sfi-NotmycHis, which results in the addition of a hexa-histidine tag at the C-terminal end of the scFv. 200 ml cultures of *E.coli* TG1 harboring one of the C6.5 mutant phagemids were grown, expression of scFv induced

with IPTG (De Bellis and Schwartz (1990), *supra*) and the culture grown at 25°C overnight. ScFv 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 mNaCl, 20 mM imidazole) and then filtered through a 0.2 micron filter.

ScFv 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 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, kinetics, and cell surface retention

The K_d of light chain shuffled C6.5 mutants isolated from phage selection using Immunotubes (Nunc) were determined by Scatchard analysis. All assays were performed in triplicate. Briefly, 50 mg of radioiodinated scFv was added to 5 x 10^6 SK-OV-cells in the presence of increasing concentrations of unlabeled scFv 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 scFv 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). The K_d of all the other isolated scFv 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 ug/ml in 10 mM sodium acetate, pH 4.5) was coupled to a CM5 sensor chip (Johnsson *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. K_{on} was determined from a plot of (l(dR/dt))/t vs concentration (Karlsson *et al.* (1991), *supra*). K_{off} was determined from the dissociation part of the sensorgram at the highest concentration of scFv analyzed. Cell surface retention of C6.5 and C6L1 was determined as described in Example 1.

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) *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_H gene repertoires (FR1 to FR3) and cloning sites for inserting the V_H CDR3FR4, single chain linker, and light chain gene from a binding scFv as a BssHII-NotI fragment. Three heavy chain shuffling libraries were created (pHEN-1-V_H1rep, pHEN-1-V_H3rep, and pHEN-1-V_H5rep), each enriched for V_H1, V_H3, or V_H5 genes by using PCR primers designed to anneal to the consensus sequence of the 3' end of V_H1, V_H3, or V_HFR3 (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 5.0×10^5 clones for pHEN-1-V_H1rep, 1.0×10^6 clones for pHEN-1-V_H3rep and 1.5×10^6 clones for pHEN-1-V_H5rep 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_H CDR3, FR4, linker, and light chain genes into the previously created V_H1. V_H3, or V_H5 repertoire using the BssHII and NotI restriction sites. After transformation, libraries of $1.0\text{-}2.0 \times 10^6$ 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_H5 library expressed scFv which bound c-erbB-2. 0/92 clones selected at random from the V_H1 or V_H repertoire expressed scFv which bound c-erbB-2.

To facilitate light chain shuffling, a library was constructed in pHEN-1 containing human V_k and V_l gene repertoires, single chain linker DNA, and cloning sites for inserting the V_H gene from binding scFv as an NcoI-XhoI fragment. The resulting library (pHEN-1-$V_l$rep) consisted of 4.5×10^6 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_H gene into pHEN-1-$V_l$rep. After transformation a library of 2.0 x 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 which bound c-erbB-2.

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).

Table 8. Frequency of binding sFv and percent of binding sFv with slower k_{off} than C6.5. Binding was determined by ELISA. k_{off} was determined by BIAcore on unpurified sFv in bacterial periplasm.

Library and method of selection	ELISA Round of selection			sFv with slower k_{off} than C6.5 (parental sFv) 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, 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=1.0×10^{-9} M

Table9. IC_{50} and K_d of C6.5 sFv 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	2.0×10^{-8}	2.0×10^{-8}
C6VLB	1.0×10^{-8}	3.0×10^{-8}
C6VLD	5.8×10^{-9}	2.6×10^{-8}
C6VLE	2.8×10^{-9}	7.1×10^{-8}
C6VLF	7.5×10^{-9}	7.9×10^{-8}

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	60	70	80	90	100
C6.5	QSVLTQPPSVSAAPGQKVTISC		SGSSSNIGNNYVS	WYQQLPGTAPKLLIY		GHTNRPA	GVPDRFSGSKSGTSASLAISGFRSEDEADYYC			AAWDDSLSG WV	FGGGTKLTVLG

Light chain shuffled mutants selected on polystyrene adsorbed antigen

C6VLB	----------		----------		----------	SDNQ--S	----------		----------		
C6VLD	----------		--TN-------		----------	TNDQ--S	----------	L----------	--------H--		
C6VLE	----------A---------		-----S--R----	-N------ -----W		RNNQ--S	----------	LQ---------	-------N- -M		
C6VLF	---------M----------		----------	----F---------H		DNNK--S	-I---I----------	V----------	-S--N---- --		
								LQ-D-------	-------N- --		

Light chain shuffled mutant selected on biotinylated antigen

C6L1	----------G----W-----		----------		----------	DNNK--S	---------Q----------L----------			---------- --	----------

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

Positive clones were ranked by IC50 as determined by competition ELISA (Table 9). Sixteen scFv with IC50s less than the IC50 of the parental scfv 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 Scatchard analysis.

Despite their lower IC50s, none of these 4 scfv had a higher affinity for c-erbB-2 (Table 9). Gel filtration analysis of the four purified scfv demonstrated the presence of two species, with size consistent for monomeric and dimeric scfv. In contrast, the parental scfv existed only as monomer.

As a result of these observations, we hypothesized that selection on immobilized antigen favored the isolation of lower affinity dimeric scfv which could achieve a higher apparent affinity due to avidity. In addition, determination of IC50 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), *supra*). To screen ELISA positive scfv for improved binding to c-erbB-2, we used a BIAcore. Periplasm preparations containing unpurified native scfv 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/R0) vs t, the presence of multiple k_{off} can be detected, indicating the presence of mixtures of monomeric and dimeric scfv. This strategy of selecting on antigen in solution, followed by BIAcore screening of ELISA positive scfv, 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 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 scfv. 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_{off} than the parental scfv. Sequencing of the V_L gene of ten of these scfv revealed one unique scfv (C6L1) (Table 10). The V_λ gene of C6L1 was derived from the same germline gene as the parental scfv, but had 9 amino acid substitutions. The C6L1 gene was subcloned and the scfv purified bIMAC and gel filtration. C6L1 scfv 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_{on} and a slower k_{off} (Table 11). The slower k_{off} was associated with a three fold increase in the retention of scfv on the surface of SK-OV-3 cells (28% at 30 minutes for C6L1 compared to 10% at 3 minutes for the parental scfv).

Table 11. Affinities and binding kinetics of c-erbB-2 binding SFv, K_d, K_{on} and K_{off} were determined by surface plasmon resonance in a BIAcore. Combined SFv 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_{on} ($M^{-1}s^{-1}$)	K_{off} ($M^{-1}s^{-1}$)
Parental C6.5	1.6×10^{-8}	4.0×10^{-5}	6.3×10^{-3}
Light Chain Shuffled C6L	2.6×10^{-9}	7.8×10^{-5}	2.0×10^{-3}
Heavy Chain Shuffled C6H1 C6H2	 5.9×10^{-9} 3.1×10^{-9}	 1.1×10^{-6} 8.4×10^{-5}	 6.2×10^{-3} 2.6×10^{-3}
Combined SFv C6H1L1 C6H2L1	 1.5×10^{-8} 6.0×10^{-9}	 4.1×10^{-5} 3.0×10^{-5}	 6.2×10^{-3} 1.8×10^{-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, 5nM, 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_{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 4th round of

selection, and 12% had a slower k_{off} than the parental scfv. No binders were isolated from either the V_H1 or V_H3 shuffled libraries. Sequencing of the V_H gene of all slower k_{off} clones revealed two unique scfv, C6H1 and C6H2 (Table 12). The V_H gene of

Table 12D **Deduced protein sequences of heavy 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		40	50 60	70	80 abc	90	100	103	100
C6.5	QVQLLQSGAELKKPGESLKISCKGSGYSFT			SYWIA	WVRQMPGKGLEYMG	LIYPGDSDIKYSPSFQG	QVTISVDKSVSTAYLQWSSLKPSDSAVYFCAR			HDVGYCSSSNCAKWPEYFQH	WGQGTLV	
Heavy Chain shuffled mutants selected on high concentration biotinylated antigen												
C6VHA2	----V---G-M----------L--D--			T----	----------	----------	----------R---T-M-Y---			----------	----------	
C6VHB2	----Q---G-M----------L--D--			T----	----------	----------	-----A-E-I-----E-----A--T-M-Y---			----------	----------	
C6VHC2	----Q---G-M----------E---S			T----	----------	----------	----------T-------R---T---Y---			----------	----------	
C6VHD2	----VE----M----------F--D-S			T----	----------	----------	----------T-M-Y---			----------	----------	
C6VHE2	----VE--G-M---R---------L--D--			T----	----------	----------	----------R---T-M-Y---			----------	----------	
C6VHF2	----VE--G-M----------L--D-S			T----	----------	----------	----------R---T-M-Y---			----------	----------	
C6VHG2	----VE--G-M----------L--D--			T----	----------	----------	----------R---T-M-Y---			----------	----------	
C6VHH2	----VE----M----------F--D-S			T----	----------	----------	----------R---T-M-Y---			----------	----------	
C6VHA3	----V---G-M----------L--D--			T----	----------	----------	-----A-E-I-----E-----A--T-M-Y---			----------	----------	
C6VHB3	----V---G-M----------L--D-S			T----	----------	----------	----------R---T-M-Y---			----------	----------	
C6VHC3	----Q---G-M----------L--D--			T----	----------	----------	-------R-I-------T---A--T-M-Y---			----------	----------	
C6VHD3	----Q---G-M----------L--D--			T----	----------	--------IR-----E-	----------R---T-M-Y---			----------	----------	
C6VHE3	----VE--G-M---R---------L--D--			T----	----------	----------	----------R---T-M-Y---			----------	----------	
C6VHF3	----------M----------F--D-S			T----	----------	----------	----------T-M-Y---			----------	----------	
C6VHG3	----------V-------Q--------D--			T-Y--	----------	I------R-I-------	--R--A----------A--T-M-Y---			----------	----------	
C6VHH3	-----E----V-E--Q--------F--D-S			T----	----------	----------	----------T-M-Y---			----------	----------	
Heavy Chain shuffled mutants selected on lower concentration biotinylated antigen												
C6H1	----VE----V----------			(E)								
C6H2	----V-----V----------			-----	----------	----A--K-I-------	----------T-M-Y---			----------	----------	
							----------T--T-M-Y---			----------	----------	

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

C6H1 and C6H2 were derived from the same germline gene as the parental scfv, 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 scfv were subcloned and purified by IMAC and gel filtration. Both scfv 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(5.9×10^{-9} M) was due to a faster k_{on}, whereas the increased affinity of C6H2 (3.1×10^{-9} M) was due to both a faster k_{on} and slower k_{off} (Table 11).

Location of mutations in chain shuffled scf

Mutations in chain shuffled scfv 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_λ 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 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_H framework 3 (V89M and F91Y), no mutations were located in residues which form the two 5 stranded ß-sheets that form the V_H-V_L 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 scfv which formed mixtures of monomer and dimer had mutations in residues which formed the ß-sheet that packs on the V_H domain (Table 4 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 V_H and V_L genes from higher affinity scfv were combined into the same scfv. Combining the V_L gene from C6L1 with the V_H gene from C6H1 resulted in an scfv (C6H1L1) with lower affinity than either C6L1 or C6H2 (Table 11). No additional reduction in k_{off} was achieved, and the k_{on} was reduced approximately 2 fold. Similarly, combining the V_L gene from C6L1 with the V_H gene from C6H2 resulted in an scfv (C6H2L1) with lower affinity than C6L1 or C6H2 (Table 11). No additional reduction in k_{off} was achieved, and the k_{on} was

reduced approximately 2 fold. Thus, in both instances, combining the independently isolated higher affinity V_H and V_l genes had a negative effect on affinity.

Example 3

Production of Higher Affinity Mutants

In order to prepare higher affinity mutants derived from C6ML3-9, part of 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 DVGY, SSSN, AKPE and YFQH respectively as described. Only the library where SSN was randomized has been characterized. A number of higher affinity mutants are listed in Table 13. below.

Table 13. Binding affinity (K_d) and K_{off} of C6 antibodies derived from C6ML3-9 having a randomized heavy chain CDR3. Altered amino acids are shown underlined.

Clone Name	CDR3 sequence	K_d (M)	K_{off} (s^{-1})
C6MH3-B1	HDVGYCTDRTCAKWPEYFQH	1.6×10^{-10}	6.7×10^{-5}
C6MH3-B15	HDVGYCESSRCAKWPEYFQH	7.7×10^{-10}	2.9×10^{-4}
C6MH3-B11	HDVGYCSDRSCAKWPEYFQH	2.2×10^{-10}	2.3×10^{-4}
C6MH3-B9	HDVGYCKTAACAKWPEYFQH	8.7×10^{-10}	3.3×10^{-4}
C6MH3-B8	HDVGYC*TERCAKWPEYFQH	7.2×10^{-10}	2.9×10^{-4}
C6MH3-B5	HDVGYCTDATCAKWPEYFQH	5.3×10^{-10}	2.3×10^{-4}
C6MH3-B2	HDVGYCTDPRCAKWPEYFQH	3.1×10^{-9}	3.1×10^{-4}
C6MH3-B39	HDVGYCTDPTCAKWPEYFQH	3.2×10^{-10}	1.9×10^{-4}
C6MH3-B25	HDVGYCLTTRCAKWPEYFQH	3.6×10^{-10}	1.9×10^{-4}
C6MH3-B21	HDVGYCTTPLCAKWPEYFQH	7.3×10^{-10}	2.4×10^{-4}
C6MH3-B20	HDVGYCSPARCAKWPEYFQH	8.7×10^{-10}	1.6×10^{-4}
C6MH3-B16	HDVGYCADVRCAKWPEYFQH	3.1×10^{-10}	2.8×10^{-4}

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 Vl CDR3 mutant library was selected on c-erbB-2, and a number of different elution conditions studied (infecting directly off of magnetic beads, 100 mM HCl, 50 mM HCl, 10 mM HCl, 2.6 M $MgCl_2$, 4 M $MgCl_2$, 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 $MgCl_2$. 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 14 (in this experiment 4 mM $MgCl_2$ was not examined).

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_H CDR3 elutions phage were eluted sequentially with 4 mM $MgCl_2$ 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 14. Results of C6.5 L3 randomization 4th round off-rate selection and elution. Underlines indicate mutated amino acids.

Clones	F	CDR3 Sequence	K_d (M)	K_{off} (s^{-1})
C6.5		AAWDDSLSGWV	1.6×10^{-8}	6.3×10^{-3}
Elution with 100 mM HCl:				
C6ML3-5	4	AAWDYSLSGWV	3.7×10^{-9}	6.3×10^{-3}
C6ML3-9		ASWDYTLSGWV	1.0×10^{-9}	1.9×10^{-4}
C6ML3-14	2	AAWDDPLWGWV	1.1×10^{-9}	7.6×10^{-4}
C6ML3-15		AAWDRPLWGWV	2.2×10^{-9}	7.7×10^{-3}
Elution with 2.6 M $MgCl_2$:				
C6ML3-5	2	AAWDYSLSGWV	3.7×10^{-9}	1.9×10^{-3}
C6ML3-7	2	AAWDYAVSGWV	2.6×10^{-9}	1.7×10^{-3}
C6ML3-12		AAWDYSRSGWV	1.6×10^{-9}	7.2×10^{-4}
C6ML3-16	2	ASWDYYRSGWV	5.0×10^{-9}	1.7×10^{-3}
C6Ml3-15		AAWDRPLWGWV	2.2×10^{-9}	1.3×10^{-3}
Elution with 100 mM tirethylamine:				
C6ML3-5	3	AAWDYSLSGWV	3.7×10^{-9}	1.9×10^{-3}
C6ML3-12	2	AAWDYSRSGWV	1.6×10^{-9}	7.2×10^{-4}
C6ML3-18		ASWDASLWGWV	2.4×10^{-9}	6.2×10^{-4}
C6ML3-19		ASWDRPLWGWV	1.5×10^{-9}	1.0×10^{-3}
C6ML3-20		AAWEQSLWGWV	3.0×10^{-9}	1.4×10^{-3}
Elution with 10 mM HCl:				
C6ML3-5		AAWDYSLSGWV	3.7×10^{-9}	1.9×10^{-3}
C6ML3-7		AAWDYAVSGWV	2.6×10^{-9}	1.7×10^{-3}
C6ML3-21		AAWDYSQSGWV	4.5×10^{-9}	2.2×10^{-3}
C6ML3-22		AAWDASLSGWV	8.3×10^{-9}	3.6×10^{-3}
C6ML3-23		ASWDHSLWGWV	1.5×10^{-9}	1.0×10^{-3}
C6ML3-24		AAWDEQIFGWV	12.4×10^{-9}	7.9×10^{-3}
C6ML3-25		AAWDNRHSGWV	7.4×10^{-9}	4.4×10^{-3}
C6ML3-26		AAWDDSRSGWV	8.3×10^{-9}	5.0×10^{-3}
Elution with 50 mM HCl:				
C6ML3-6		ASWDYSLSGWV	3.2×10^{-9}	1.9×10^{-3}
C6ML3-7		AAWDYAVSGWV	2.6×10^{-9}	1.7×10^{-3}
C6ML3-12		AAWDYSRSGWV	1.6×10^{-9}	7.2×10^{-4}
C6ML3-17		ASWDYYRSGWV	5.0×10^{-9}	1.7×10^{-3}

C6ML3-27		TAWDYSLSGWV	no expression	
C6ML3-28		ASWDYALSGWV	2.5×10^{-9}	1.7×10^{-3}
C6ML3-29		AAWDGTLWGWV	1.7×10^{-9}	2.2×10^{-3}
Elution with 1 μM c-erbB-2 ECD for 30 minutes				
C6ML3-5	5	AAWDYSLSGWV	3.7×10^{-9}	1.9×10^{-3}
C6ML3-17		AAWDYALSGWV	no expression	
C6ML3-30	3	ASWDYYLIGWV	no expression	

Table15 Sequences of primers used.

LMB3	5'-CAGGAAACAGCTATGAC-3'
fd-seq1	5'-GAATTTTCTGTATGAGG-3'
PHEN-1seq	5'-CTATGCGGCCCCATTCA-3'
Linkseq	5'-CGATCCGCCACCGCCAGAG-3'
PVH1For1	5'-TCGCGCGCAGTAATACACGGCCGTGTC-3'
PVH3For1	5'-TCGCGCGCAGTAATACACAGCCGTGTCCTC-3'
PVH5For1	5'-TCGCGCGCAGTAATACATGGCGGTGTCCGA-3'
PVH1For2	5'-GAGTCATTCTCGACTTGCGGCCGCTCGCGCGCAGTAATACACGGCCGTGTC-3'
PVH3For2	5'-GAGTCATTCTCGACTTGCGGCCGCTCGCGCGCAGTAATACACAGCCGTGTCCTC-3'
PVH5For2	5'-GAGTCATTCTCGACTTGCGGCCGCTCGCGCGCAGTAATACATGGCGGTGTCCGA-3'
PC6VL1Back	5'-AGCGCCGTGTATTTTGCGCGCGACATGACGTGGGATATTGC-3'
RJH1/2/6Xho	5'-ACCCTGGTCACCGTCTCGAGTGGTGGA-3'
RJH3Xho	5'-ACAATGGTCACCGTCTCGAGTGGTGGA-3'
RJH4/5Xho	5'-ACCCTGGTCACCGTCTCGAGTGGTGGA-3'
PC6VH1For	5'-GAGTCATTCTCGTCTCGAGACGGTGACCAGGGTGCC-3'

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.

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WHAT IS CLAIMED IS:

1. A human antibody that specifically binds to c-erbB-2, said antibody being a C6 antibody.

2. The antibody of claim 1, wherein said antibody has the variable heavy (V_H) chain of C6.5.

3. The antibody of claim 1, wherein said antibody has the variable light (V_H) chain of C6.5.

4. The antibody of claim 1, wherein said antibody is C6.5.

5. The antibody of claim 1, wherein said antibody has the amino acid sequence of C6.5.

6. The antibody of claim 1, wherein said antibody has the amino acid sequence of C6ML3-14.

7. The antibody of claim 1, wherein said antibody has the amino acid sequence of C6L1.

8. The antibody of claim 1, wherein said antibody has the amino acid sequence of C6MH3-B1.

9. The antibody of claim 1, wherein said antibody has the amino acid sequence of C6ML3-9.

10. The antibody of claim 1, wherein said antibody is an Fab.

11. The antibody of claim 1, wherein said antibody is an $(Fab')_2$.

12. The antibody of claim 1, wherein said antibody is an $(SFv')_2$.

13. The antibody of claim 1, wherein said antibody is C6.5Fab.

14. The antibody of claim 1, wherein said antibody is C6.5(Fab')$_2$.

15. The antibody of claim 1, wherein said antibody is C6.5(SFv')$_2$.

16. The antibody of claim 1, wherein said antibody has a K_d ranging from about 1.6×10^{-8} M to 1.0×10^{-11} M in SK-BR-3 using a Scatchard assay or against purified c-erbB-2 by surface plasmon resonance in a BIAcore.

17. The antibody of claim 16, wherein said K_d is about 1.6×10^{-8} M.

18. A nucleic acid encoding a human C6 antibody that specifically binds to c-erbB-2.

19. The nucleic acid of claim 18, wherein said C6 antibody binds to SK-BR-3 cells with a K_d less than about 1.6×10^{-8} as determined using a scatchard assay.

20. The nucleic acid of claim 18, wherein said nucleic acid encodes the variable light (V_L) chain of C6.5.

21. The nucleic acid of claim 18, wherein said nucleic acid encodes the variable heavy (V_H) chain of C6.5.

22. The nucleic acid of claim 18, wherein said nucleic acid encodes C6.5.

23. The nucleic acid of claim 18, wherein said nucleic acid encodes the the amino acid of a C6.5 antibody and conservative amino acid substitutions of said C6.5 antibody.

24. A cell comprising a recombinant nucleic acid that encodes a human antibody that specifically binds c-erbB-2, wherein said antibody is a C6 antibody.

25. A chimeric molecule that specifically binds a tumor cell bearing c-erbB-2, said chimeric molecule comprising an effector molecule attached to a human C6 antibody that specifically binds c-erbB-2.

26. The chimeric molecule of claim 25, wherein said C6 antibody is a single chain Fv (sFv).

27. The chimeric molecule of claim 25, wherein said effector molecule is selected from the group consisting of a cytotoxin, a label, a radionuclide, a drug, a liposome, a ligand, and an antibody.

28. The chimeric molecule of claim 25, wherein said effector molecule is a *Pseudomonas* exotoxin.

29. The chimeric molecule of claim 25, wherein said chimeric molecule is a fusion protein.

30. A method for making a C6 antibody, said method comprising:

i) providing a phage library presenting a C6.5 variable heavy (V_H) chain and a multiplicity of human variable light (V_L) chains;

ii) panning said phage library on c-erbB-2; and

iii) isolating phage that specifically bind said c-erbB-2.

31. The method of claim 30, further comprising:

iv) providing a phage library presenting a the variable light chain (V_L) of the phage isolated in step iii and a multiplicity of human variable heavy (V_H) chains;

v) panning said phage library on immobilized c-erbB-2; and

vi) isolating phage that specifically bind said c-erbB-2.

32. A method for making a C6 antibody, said method comprising:

i) providing a phage library presenting a C6.5 variable light (V_L) chain and a multiplicity of human variable heavy (V_H) chains;

ii) panning said phage library on immobilized c-erbB-2; and

iii) isolating phage that specifically bind said c-erbB-2.

33. A method for making a C6 antibody, said method comprising:

i) providing a phage library presenting a C6.5 variable light (V_L) and a C6.5 variable heavy chain encoded by a nucleic acid variable in the sequence encoding the CDRs such that each phage display a different CDR;

ii) panning said phage library on c-erbB-2; and

iii) isolating phage that specifically bind said c-erbB-2.

34. A method for impairing growth of tumor cells bearing c-erbB-2, said method comprising contacting said tumor with a chimeric molecule comprising a cytotoxin attached to a human C6 antibody that specifically binds c-erbB-2.

35. A method for detecting tumor cells bearing c-erbB-2, said method comprising contacting said tumor with a chimeric molecule comprising a label attached to a human C6 antibody that specifically binds c-erbB-2.

36. A polypeptide comprising one or more of the complementarity determining regions (CDRs) whose amino acid sequence is

selected from the group consisting of the CDRs listed in the Specification and Examples 1, 2 and 3.

37. A nucleic acid molecule comprising a nucleotide sequence encoding a single chain polypeptide that exhibits the antibody-binding specificity of a human C6 antibody, said polypeptide comprising:

a) a first polypeptide domain, comprising an amino acid sequence that is homologous to the binding portion of a variable region of a heavy chain of a human C6 antibody;

b) a second polypeptide domain, comprising an amino acid sequence that is homologous to the binding portion of a variable region of a light chain of a human C6 antibody; and

c) at least one polypeptide linkers comprising an amino acid sequence spanning the distance between the C-terminus of one of the first or second domains and the N-terminus of the other, whereby said linker joins the first and second polypeptide domains into a single chain polypeptide.

38. A polypeptide which exhibits immunological binding properties of a human C6 antibody, said polypeptide comprising first and second domains connected by a linker moiety, wherein:

a) the first domain comprises at least one amino acid sequence that is homologous to a CDR derived from a heavy chain of a human C6 antibody; and

b) the second domain comprises at least one amino acid sequence that is homologous to a CDR derived from a light chain of a human C6 antibody.

39. The polypeptide of claim 38, wherein the first domain comprises a group of amino acid residues that are homologous to a set of CDRs derived from a heavy chain of a human C6 antibody.

40. The polypeptide of claim 38, wherein the second domain comprises a group of amino acid residues that are homologous to a set of CDRs derived from a light chain of a human C6 antibody.

41. An expression cassette, comprising:

a) the nucleic acid molecule of claim 35; and

b) a control sequence operably linked to the nucleic molecule and capable of directing the expression thereof.

42. An expression cassette, comprising:

a) the nucleic acid molecule of claim 35; and

b) a control sequence operably linked to the nucleic molecule and capable of directing the expression thereof.

43. An expression cassette, comprising:

a) the nucleic acid molecule of claim 36; and

b) a control sequence operably linked to the nucleic molecule and capable of directing the expression thereof.

44. A method of inducing the production of a polypeptide, comprising:

a) introducing the expression cassette of claim 42 into a host cell whereby the cassette is compatible with the host cell and replicates in the host cell;

b) growing the host cell whereby the polypeptide is expressed; and

c) isolating the polypeptide.

45. A method of inducing the production of a polypeptide, comprising:

a) introducing the expression cassette of claim 42 into a host cell whereby the cassette is compatible with the host cell and replicates in the host cell;

b) growing the host cell whereby the polypeptide is expressed; and

c) isolating the polypeptide.

46. A method of inducing the production of a polypeptide, comprising:

a) introducing the expression cassette of claim 42 into a host cell whereby the cassette is compatible with the host cell and replicates in the host cell;

b) growing the host cell whereby the polypeptide is expressed; and

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.

erb6 (C6.5) Translated Sequence
Thursday, January 25, 1996 11:36 AM

Sequence Range: 1 to 774

```
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     *    *    *      *    *      *    *      *
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GTC CAC GTC GAC AAC GTC AGA CCC CGT CTC AAC TTT TTT GGG CCC CTC
 Q   V   Q   L   L   Q   S   G   A   E   L   K   K   P   G   E>

50          60          70          80          90
 *    *    *    *      *    *      *    *      *    *
TCT CTG AAG ATC TCC TGT AAG GGT TCT GGA TAC AGC TTT ACC AGC TAC
AGA GAC TTC TAG AGG ACA TTC CCA AGA CCT ATG TCG AAA TGG TCG ATG
 S   L   K   I   S   C   K   G   S   G   Y   S   F   T   S   Y>

  100         110         120         130         140
    *    *      *    *      *    *      *    *      *
TGG ATC GCC TGG GTG CGC CAG ATG CCC GGG AAA GGC CTG GAG TAC ATG
ACC TAG CGG ACC CAC GCG GTC TAC GGG CCC TTT CCG GAC CTC ATG TAC
 W   I   A   W   V   R   Q   M   P   G   K   G   L   E   Y   M>

    150         160         170         180         190
 *    *      *    *      *    *      *    *      *    *
GGG CTC ATC TAT CCT GGT GAC TCT GAC ACC AAA TAC AGC CCG TCC TTC
CCC GAG TAG ATA GGA CCA CTG AGA CTG TGG TTT ATG TCG GGC AGG AAG
 G   L   I   Y   P   G   D   S   D   T   K   Y   S   P   S   F>

        200         210         220         230         240
  *    *      *    *      *    *      *    *      *    *
CAA GGC CAG GTC ACC ATC TCA GTC GAC AAG TCC GTC AGC ACT GCC TAC
GTT CCG GTC CAG TGG TAG AGT CAG CTG TTC AGG CAG TCG TGA CGG ATG
 Q   G   Q   V   T   I   S   V   D   K   S   V   S   T   A   Y>

          250         260         270         280
     *    *      *    *      *    *      *    *      *
TTG CAA TGG AGC AGT CTG AAG CCC TCG GAC AGC GCC GTG TAT TTT TGT
AAC GTT ACC TCG TCA GAC TTC GGG AGC CTG TCG CGG CAC ATA AAA ACA
 L   Q   W   S   S   L   K   P   S   D   S   A   V   Y   F   C>

290         300         310         320         330
 *    *      *    *      *    *      *    *      *    *
GCG AGA CAT GAC GTG GGA TAT TGC AGT AGT TCC AAC TGC GCA AAG TGG
CGC TCT GTA CTG CAC CCT ATA ACG TCA TCA AGG TTG ACG CGT TTC ACC
 A   R   H   D   V   G   Y   C   S   S   S   N   C   A   K   W>

  340         350         360         370         380
    *    *      *    *      *    *      *    *      *
CCT GAA TAC TTC CAG CAT TGG GGC CAG GGC ACC CTG GTC ACC GTC TCC
GGA CTT ATG AAG GTC GTA ACC CCG GTC CCG TGG GAC CAG TGG CAG AGG
 P   E   Y   F   Q   H   W   G   Q   G   T   L   V   T   V   S>

    390         400         410         420         430
 *    *      *    *      *    *      *    *      *    *
TCA GGT GGA GGC GGT TCA GGC GGA GGT GGC TCT GGC GGT GGC GGA TCG
AGT CCA CCT CCG CCA AGT CCG CCT CCA CCG AGA CCG CCA CCG CCT AGC
 S   G   G   G   G   S   G   G   G   G   S   G   G   G   G   S>

        440         450         460         470         480
  *    *      *    *      *    *      *    *      *    *
CAG TCT GTG TTG ACG CAG CCG CCC TCA GTG TCT GCG GCC CCA GGA CAG
GTC AGA CAC AAC TGC GTC GGC GGG AGT CAC AGA CGC CGG GGT CCT GTC
 Q   S   V   L   T   Q   P   P   S   V   S   A   A   P   G   Q>
```

```
          490         500         510         520
      *       *   *       *   *       *   *       *
AAG GTC ACC ATC TCC TGC TCT GGA AGC AGC TCC AAC ATT GGG AAT AAT
TTC CAG TGG TAG AGG ACG AGA CCT TCG TCG AGG TTG TAA CCC TTA TTA
 K   V   T   I   S   C   S   G   S   S   S   N   I   G   N   N>

530         540         550         560         570
  *       *   *       *   *       *   *       *   *       *
TAT GTA TCC TGG TAC CAG CAG CTC CCA GGA ACA GCC CCC AAA CTC CTC
ATA CAT AGG ACC ATG GTC GTC GAG GGT CCT TGT CGG GGG TTT GAG GAG
 Y   V   S   W   Y   Q   Q   L   P   G   T   A   P   K   L   L>

  580         590         600         610         620
    *   *       *   *       *   *       *   *       *
ATC TAT GGT CAC ACC AAT CGG CCC GCA GGG GTC CCT GAC CGA TTC TCT
TAG ATA CCA GTG TGG TTA GCC GGG CGT CCC CAG GGA CTG GCT AAG AGA
 I   Y   G   H   T   N   R   P   A   G   V   P   D   R   F   S>

    630         640         650         660         670
*       *   *       *   *       *   *       *   *       *
GGC TCC AAG TCT GGC ACC TCA GCC TCC CTG GCC ATC AGT GGG TTC CGG
CCG AGG TTC AGA CCG TGG AGT CGG AGG GAC CGG TAG TCA CCC AAG GCC
 G   S   K   S   G   T   S   A   S   L   A   I   S   G   F   R>

      680         690         700         710         720
  *       *   *       *   *       *   *       *   *       *
TCC GAG GAT GAG GCT GAT TAT TAC TGT GCA GCA TGG GAT GAC AGC CTG
AGG CTC CTA CTC CGA CTA ATA ATG ACA CGT CGT ACC CTA CTG TCG GAC
 S   E   D   E   A   D   Y   Y   C   A   A   W   D   D   S   L>

          730         740         750         760
      *       *   *       *   *       *   *       *   *
AGT GGT TGG GTG TTC GGC GGA GGG ACC AAG CTG ACC GTC CTA GGT GCG
TCA CCA ACC CAC AAG CCG CCT CCC TGG TTC GAC TGG CAG GAT CCA CGC
 S   G   W   V   F   G   G   G   T   K   L   T   V   L   G   A>

770
  *
GCC GCA
CGG CGT
 A   A>
```

Figure 2

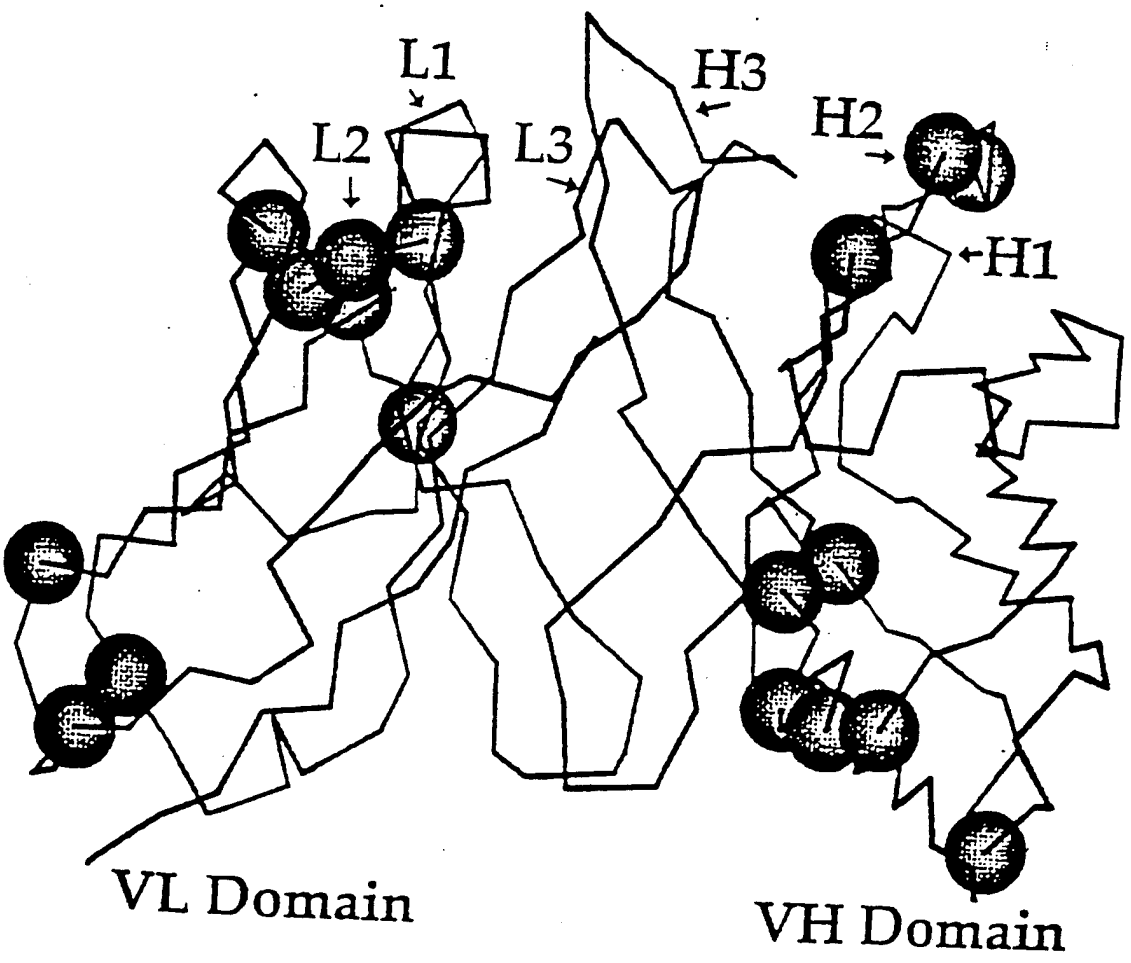

Figure 3