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13. ABSTRACT *(Maximum 200*

A major goal of cancer research has been to identify tumor antigens which are qualitatively or quantitatively different from normal cells. The presence of such antigens could be detected by monoclonal antibodies that would form the basis of diagnostic tests and therapeutic agents. For this project, we developed novel technology, phage display, to produce a new generation of antibodies. We have produced a library of human antibodies from which we can isolate panels of monoclonal antibodies to any purified antigen within 2 weeks. Methodologies have been developed to increase antibody affinity to values not achievable previously. Finally, we have developed methodologies to select libraries directly on tumor cells for the purpose of generating antibodies with desirable functional properties, such as inhibition of cell growth or induction of apoptosis. Tumor cell specific antibodies have been isolated and are being used to identify the antigens to which they bind. This should result in the identification of novel tumor antigens.

We have applied these technologies to produce a human antibody that targets the ErbB2 receptor overexpressed in one third of breast cancers. With collaborators at UCSF, we have used these antibodies to target doxorubicin containing liposomes, which can cure mice of tumors. The National Cancer Institute Decision Network is supporting further pre-clinical development for anticipated clinical trials for breast cancer.

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

    5.2 Appendix 2:

Adams GP, Schier R, Marshall K, Wolf EJ, McCall AM, Marks JD and Weiner LM. Increased affinity leads to improved selective tumor delivery of single-chain Fv antibodies. Caner Res. 58: 485-490, 1998

    5.3 Appendix 3:

Adams GP, Schier R, McCal AM, Crawford RS, Wolf EJ, Weiner LM and Marks JD. Prolonged in vivo tumour retention of a human diabody targeting the extracellular domain of HER2/neu. Brit. J. Cancer. In press.

    5.4 Appendix 4:

Sheets MD, Amersdorfer P, Finnern R, Sargent P, Lindqvist E, Schier R, Hemingsen G, Wong C, Gerhart JC and Marks JD. Efficient construction of a large non-immune phage antibody library: the production of panels of high affinity human single chain antibodies to protein antigens. Proc. Natl. Acad. Sci. USA. In press.

1.   Introduction

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

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

1.1.   Limitations of murine monoclonal antibodies

Production of monoclonal antibodies from hybridomas requires administration of an immunogenic antigen. Many of the antigens overexpressed on tumor cells are not likely to be immunogenic, since they are also present on normal cells at low levels and would be recognized as 'self antigens'. Thus an immune response would not be generated. In addition, many of the antigens are polysaccharides and do not elicit classic T-cell help needed to trigger the production of higher affinity antibodies. Consequently, many of the antibodies produced are of relatively low affinity. Even when a vigorous immune response is elicited, the affinities (K_d) of the resulting monoclonal antibodies are not likely to be better than 1.0×10^{-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 gene 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   Isolate human scFv antibody fragments which bind breast tumor antigens using a pre-existing scFv phage antibody library.

2   Create a non-immune human Fab phage antibody library containing 10^9-10^{11} members.

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.

4   Characterize binding scFv and Fabs with respect to DNA sequence, specificity, and affinity.

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.

6   Characterize mutant antibody fragments with respect to DNA sequence, specificity, and affinity.

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

2.   Body of report

The work in the third year of the grant builds directly on results produced during the first two year of funding. In addition, some of the results in last years report were either preliminary or only partially complete. For both of these reasons, I have included some results previously reported in the year 1 and year 2 reports. To facilitate distinction, each section is broken down by year, although this distinction is somewhat arbitrary.

Work during the first two years of the grant focused on selection of the smaller scFv phage antibody library on breast tumor cell lines (technical objective 1) and creation of a large phage antibody library (technical objective 2). Characterization of the large scFv phage antibody library was completed in year 3, validating the library as a source of high affinity human antibodies to protein antigens. This work is now in press (25). The major focus of work in the third year was development of selection strategies for isolating cell type specific scFv for tumor targeting and as tools for identifying novel tumor antigens (technical objectives 3 and 4).

In the first two years, work was also begun to identify the optimal means of increasing antibody fragment affinity (technical objective 5) using a human scFv (C6.5) isolated from a non-immune phage antibody library which binds the breast tumor antigen ErbB-2 (26). This work was completed in year 3. As a result of this work, we were able to develop an efficient and effective approach to create, identify, and characterize higher affinity antibody fragments in vitro (27-29). Using this approach, we have engineered the affinity of C6.5 to produce mutants with affinities between 1.0×10^{-6} M to 1.3×10^{-11} M (30). The best binders represent the highest affinity antibodies ever produced to any tumor antigen and our results provide a general approach to rapidly increase antibody affinity to values not achievable with animal immunization. The methodology for affinity maturation and the specific molecules are the subject of a patent application by the Regents of the University of California. The antibody fragments also permit for the first time examination of the relationship between affinity and specific tumor targeting using antibodies that differ by only a few amino acids in sequence and which recognize identical epitopes. Over the range of affinities examined in detail ($K_d = 3.2 \times 10^{-7}$ to 1.0×10^{-9} M) increased affinity correlated with greater quantitative retention of scFv in tumor *scid* mice bearing human SK-OV-3 tumors (31). In contrast, higher affinity scFv ($K_d = 1.6 \times 10^{-10}$ to 1.3×10^{-11} M) did not provide any additional quantitative increase in delivery or retention of scFv in tumor. We also had begun to examine the effect of size and valency on targeting using the monomeric scFv as building blocks to create larger multivalent molecules. A dimeric diabody (scFv)$_2$ was constructed from the genes of C6.5 and it's in vitro cell retention and in vivo targeting were reported. This work is in press (32). Since in vivo characterization of these molecules was not formally one of the technical objectives, we are continuing to use these molecules to study the relationship between antibody affinity, size and valency and specific tumor targeting as part of NIH R01 1 CA65559-01A1.

C6.5 molecules developed as part of this grant are also being pursued as potential therapies for breast cancer and other ErbB2 expressing tumors. In collaboration with the Bay Area Breast Cancer SPORE, C6.5 targeted stealth liposomes were generated (33, 34). When loaded with doxorubicin, the targeted liposomes have shown promising therapeutic results, including cures in tumor bearing mice (see below). These results were presented to the National Cancer Center Decision Network who decided to support further pre-clinical development at the 2A level (scale-up of production for toxicology and development of processes for GMP manufacture).

2.1. Technical Objective 1: Selection of the smaller scFv phage antibody library on breast tumor cell lines

Year 1

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. 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.

2.2.   Technical Objective 2: Creation of a non-immune human Fab phage antibody library containing 10^9-10^{11} members

Year 1

In the original grant application, we had proposed creating a large Fab phage antibody library using combinatorial infection. By the time work was begun on the project, a large Fab phage antibody library (7.0 x 10^{10} members) had already been created in the Laboratory of Dr. Greg Winter, using combinatorial infection (35). 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 x 10^{-8} to 1.0 x 10^{-8} M were isolated against protein antigens (35). 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 x 10^7member 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 (Figure 1). Human peripheral blood lymphocyte and spleen RNA was primed with immunoglobulin C_K, 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_K, and V_λ gene repertoires. The V_H gene repertoires were cloned into the vector pUC119Sfi-Not as Nco1-NotI fragments, to create a library of 8.0 x 10^8 members. The library was diverse by PCR fingerprinting. Single chain linker DNA was spliced onto the V_K, 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 7.2 x 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 x 10^9 members. The library was diverse as determined by BstN1 fingerprinting.

Years 2 and 3

To verify the quality of the library, phage were prepared and selected on 14 different protein antigens (25). The results are shown in Table 1. scFv antibodies were obtained against all antigens used for selection, with between 3 and 15 unique scFv isolated per antigen (average 8.7) (Table 1). This compares favorably to results obtained from smaller scFv libraries (1 to a few binders obtained against only 70% of antigens used for selection). Affinities of 4 anti-ErbB-2 scFv and 4 anti-Botulinum scFv were measured using surface plasmon resonance in a BIAcore and found to range from 4.0 x 10^{-9} M to 2.2 x 10^{-10} M for the anti-ErbB2 scFv and 2.6 x 10^{-8} M to 7.15 x 10^{-8} M for the anti-Botulinum scFv (Table 2). scFv were highly specific for the antigen used for selection (Figure 2). The library could also be successfully selected on complex mixtures of antigen. For example, selection on Chlamydia trachomatis elementary bodies (the causative organism of Chlamydial disease) yielded 7 scFv that specifically recognized chlamydia (Table 1 and Figure 3). The scFv could be successfully used in a number of immunologic assays including ELISA (Figure 2), immunofluorescence (Figure 3), Western blotting, epitope mapping and immunoprecipitation. The number of binding antibodies for each antigen, and the affinities of the binding scFv are comparable to results obtained from the best phage antibody libraries (Table 3). Thus the library was established as a source of panels of human antibodies against any antigen with affinities at least equivalent to the secondary murine response.
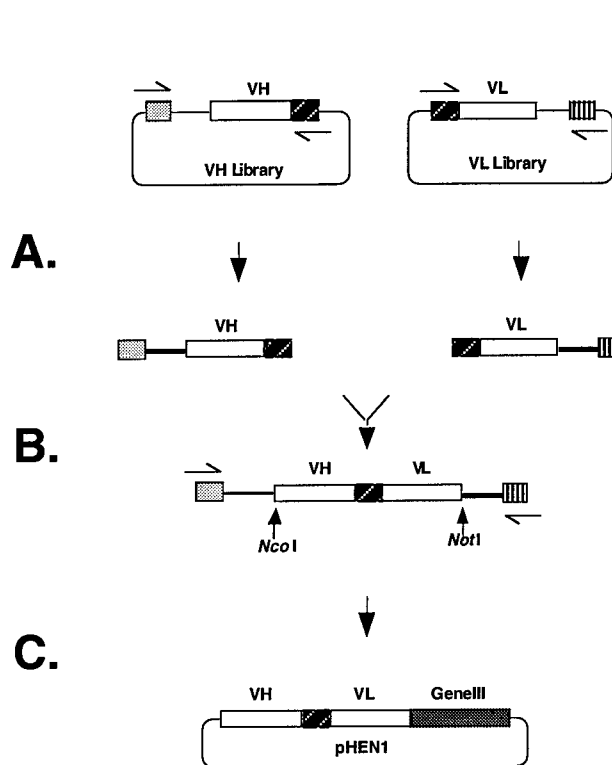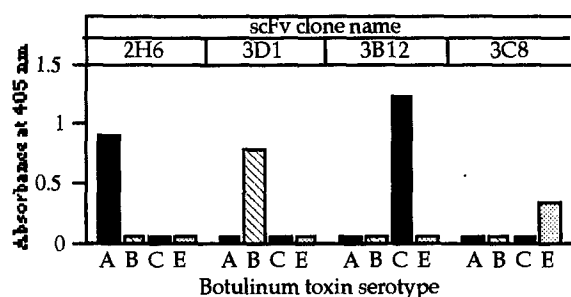
Figure 1. Method for construction of a large human scFv phage antibody library. The strategy for library construction involved optimizing the individual steps of library construction to increase both the efficiency of scFv gene assembly and to increase the efficiency of cloning assembled scFv genes. **(A)**. First, mRNA from lymphocytes was used to generate V_H and V_L gene repertoires by RTPCR which were cloned into different vectors to create V_H and V_L gene libraries of 8.0×10^8 and 7.2×10^6 members respectively. The cloned V-gene libraries provided a stable and limitless source of V_H and V_L genes for scFv assembly. DNA encoding the peptide $(G_4S)_3$ was incorporated into the 5' end of the V_L library. This permitted generation of scFv genes by PCR splicing 2 DNA fragments. Previously, scFv gene repertoires were assembled from 3 separate DNA fragments consisting of V_H, V_L and linker DNA. **(B)** V_H and V_L gene repertoires were amplified from the separate libraries and assembled into an scFv gene repertoire using overlap extension PCR. The primers used to reamplify the V_H and V_L gene repertoires annealed 200 bp upstream of the 5' end of the V_H genes and 200 bp down stream of the V_L genes. These long overhangs ensured efficient restriction enzyme digestion.**(C.)** The scFv gene repertoire was digested with NcoI and NotI and cloned into the plasmid pHEN1 as fusions with the M13 gene III coat protein gene (▧) for phage-display.

Table 1. Results of phage antibody library selections. For each antigen (column 1), the number and the percentage of positive clones selected (column 2) and the number of different antibodies isolated (column 3) is indicated.

Protein antigen used for selection	Percentage (number) of ELISA positive clones	Number of different antibodies isolated
FGF Receptor ECD	69 (18/26)	15
BMP Receptor Type I ECD	50 (12/24)	12
Activin Receptor Type I ECD	66 (16/24)	7
Activin Receptor Type II ECD	66 (16/24)	4
Erb-B2 ECD	91 (31/34)	14
VEGF	50 (48/96)	6
BoNT/A	28 (26/92)	14
BoNT-A C-fragment	95 (87/92)	10
BoNT/B	10 (9/92)	5
BoNT/C	12 (11/92)	5
BoNT/E	9 (8/92)	3
Bungarotoxin	67 (64/96)	15
Cytochrome b5	55 (53/96)	5
Chlamydia trachomatis EB	66 (63/96)	7

Table 2. Affinities and binding kinetics of anti-BoNT A C-fragment and anti-Erb-B2 scFv. Association (k_{on}) and dissociation (k_{off}) rate constants for purified scFvs were measured using surface plasmon resonance (BIAcore) and K_d calculated as (k_{off}/k_{on}).

Specificity and clone	K_d (x 10^{-9}M)	k_{on} (x 10^5M^{-1}s^{-1})	k_{off} (x 10^{-3}s^{-1})
ErbB-2 B7A	0.22	4.42	0.1
ErbB-2 G11D	0.48	2.19	0.11
ErbB-2 A11A	0.49	3.69	0.18
ErbB-2 F5A	4.03	1.62	0.65
BoNT-A 2A9	26.1	0.25	0.66
BoNT-A 2H6	38.6	2.2	8.5
BoNT-A 3F6	66.0	4.7	30.9
BoNT-A 2B6	71.5	1.1	7.8

Figure 2. Specificity of anti-Botulinum (BoNT) scFv. scFv were isolated by selection of a phage antibody library on either BoNT serotype A (2H6), serotype B (3D1), serotype C (3B12) or serotype E (3C8). Specificity was determined by ELISA on the different serotypes. Each scFv was specific for the serotype used for selection.
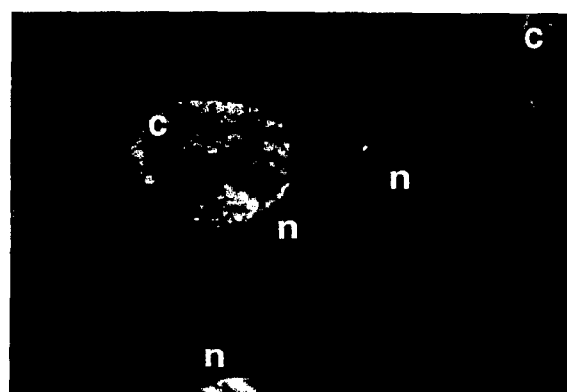
Figure 3. Specificity of anti-Chlamydial scFv. An anti-chlamydial scFv was isolated by direct selection of a phage antibody library on Chlamydial elementary bodies (EB). Purified scFv was used to stain chlamydial infected HeLa cells. c= chlamydial EB FITC stained. n = nucleus of HeLa cell.

Table 3. Comparison of protein binding antibodies selected from non-immune phage-display antibody libraries. For library type, N = V-gene repertoires obtained from V-genes rearranged in vivo; SS = semi-synthetic V-genes constructed from cloned V-gene segments and synthetic oligonucleotides encoding V_H CDR3. ND = not determined.

Library	Library size and type*	Number of protein antigens studied	Average number of antibodies per protein antigen	Number of affinities measured	Range of affinities for protein antigens K_d (x 10^{-9}M)
Marks et al (20)	3.0 x 10^7 (scFv, N)	2	2.5	1	100-2000
Nissim et al (36)	1.0 x10^8 (scFv, SS)	15	2.6	ND	ND
deKruif et al (37)	3.6 x 10^8 (scFv, SS)	12	1.9	3	100 - 2500
Griffiths et al (35)	6.5 x 10^{10} (Fab, SS)	30	4.8	3	7 - 58
Vaughan et al (38)	1.4 x 10^{10} (scFv, N)	3	7.0	3	4.2 - 8.0
Sheets et al (this work)	6.7 x 10^9 (scFv, N)	14	8.7	8	0.22 - 71.5

Significance: We have generated a high comlexity human scFv phage antibody library from which a panel of high affinity human scFv can be generated against any purified antigen. Such a library is ideal for probing the surface of cells to identify novel cell surface markers.

2.3.   Technical Objectives 3 and 4: Isolate human scFv antibody fragments which bind breast tumor antigens by selecting this new non-immune scFv phage antibody library on primary and metastatic breast tumor cell lines. Characterize binding scFv with respect to DNA sequence, specificity, and affinity.

Year 2

The 7.0×10^9 member scFv phage antibody library described in section 2.2 was selected on the malignant breast tumor cell lines MB231 and ZR-75-1, both with and without negative selections on the normal breast cell line HBL100. Similar results were obtained as described in section 2.1 above. scFv were isolated that could not distinguish malignant from non-malignant cell lines.

Year 3

To increase the specificity of selections, it was hypothesized that phage binding cell surface receptors could be taken up into cells by receptor mediated endocytosis and could then be recovered from cells by lysing the cells (39). This assumed: 1) that phage could be internalized by receptor mediated endocytosis and 2) that phage could be recovered in the infectious state from within cells prior to lysosomal degradation. The ability to select for internalized phage antibodies would have two major benefits: 1) the identification of antibodies that bind to receptors capable of internalization and 2) an added level of specificity in the selection process. Identification of antibodies which are internalized would be highly useful for many targeted therapeutic approaches where internalization is essential (e.g. immunotoxins, targeted liposomes, targeted gene therapy vectors and others).

2.3A.   Receptor mediated internalization of C6.5 phage

To determine proof of principle, we utilized C6.5 phage and C6.5 diabody phage. We have previously shown that C6.5 scFv is internalized, but at a slow rate, and that the C6.5 diabody is somewhat better internalized (probably because it causes receptor dimerization). C6.5 phage, C6.5 diabody phage or an irrelevant anti-Botulinum phage were incubated with SKBR3 cells (ErbB2 expressing breast tumor cell line) at either 37° C or 4° C and non-internalized phage removed by sequential washing with PBS and low glycine buffer. The cells were then permeabilized and biotinylated anti-M13-antibody added followed by streptavidin Texas Red. Cells were then examined by using a confocal microscope. Both C6.5 phage and C6.5 diabody phage were observed within the cytoplasm (Figure 4). Approximately 1% of cells had internalized C6.5 phage and 20% of the cells had internalized C6.5 diabody phage. There was no internalization of the anti-Botulinum phage (Figure 4).

To determine if infectious phage could be specifically taken up and recovered from within cells, C6.5 phage or C6.5 diabody phage were incubated with SKBR3 cells at 37° C. Non bound phage were removed by washing with PBS and phage bound to the cell surface were eluted by washing twice with low pH glycine. The cells were then lysed and each fraction (the first and second glycine washes and the cytoplasmic fraction) used to infect E. coli TG1. Twenty times (C6.5) or 30 times (C6.5 diabody) more phage were bound to the cell surface than the anti-Botulinum phage (glycine 1 wash) (Table 4 and Figure 5.). After the second glycine wash, the titre of infectious phage from the cell surface decreased, indicating that washing was effective at removing surface bound phage (Table 4 and Figure 5.). After cell lysis, the titer increased more than 10 fold (C6.5 phage) or 50 fold (C6.5 diabody phage) from the second glycine wash. We believe this titre represents phage recovered from inside the cell. Recovery of phage from inside the cell was 100 times higher for ErbB2 binding C6.5 than for anti-Botulinum phage and 200 fold higher for C6.5 diabody phage (Table 4 and Figure 5).

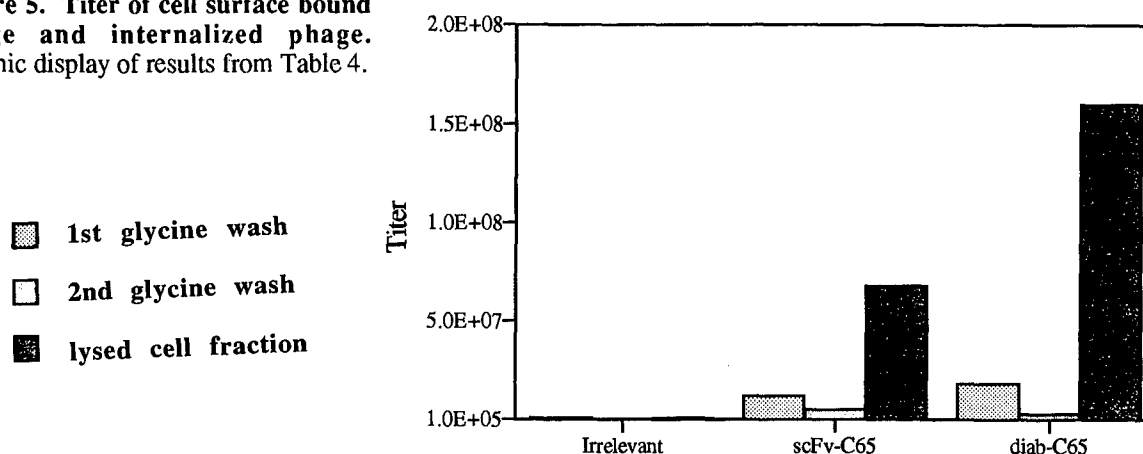|	Anti-botulinum phage	C6.5 phage	C6.5 diabody phage
A.			
B.			
C.			
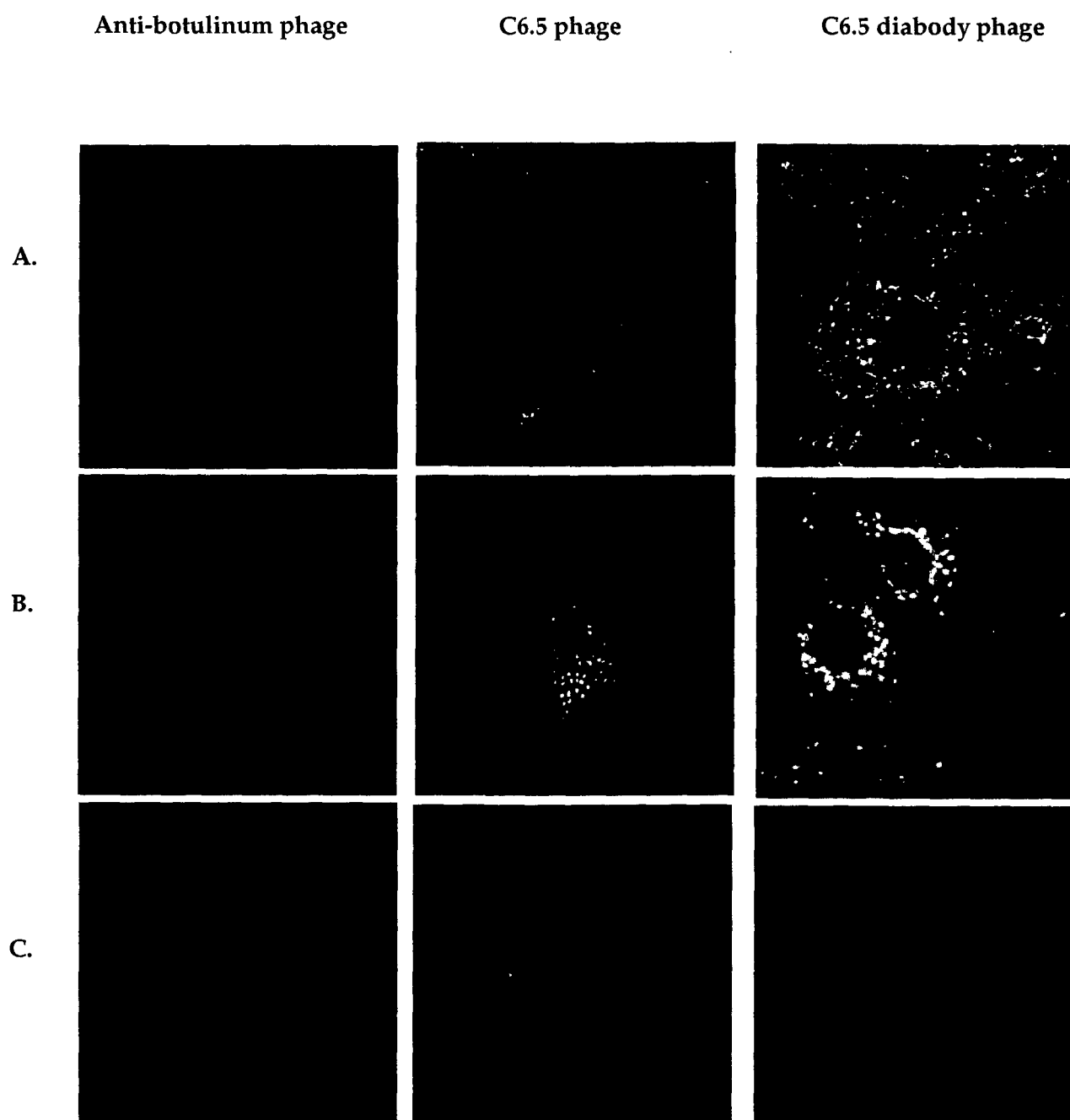
Figure 4. Internalization of phage as determined by immunofluorescence and confocal microscopy. SKBR3 cells were incubated with anti-Botulinum phage (left panels), C6.5 phage (middle panels) or C6.5 diabody phage (left panels) at 37°C (top two rows A and B) or at 4°C (bottom row C). Cells were washed with PBS (top row A) to remove unbound phage antibody, followed by low pH glycine (middle row B and bottom row C) to remove surface bound phage antibody. Phage antibodies were visualized using biotinylated anti-M13 antibody and streptavidin Texas red.

Table 4. Titer of cell surface bound phage and internalized phage. 5.0×10^{11} phage (anti-Botulinum or anti-ErbB2) were incubated with approximately 1.0×10^5 ErbB2 expressing SKBR3 cells at 37°C. Cells were washed 10 times with PBS and surface bound phage eluted with two low pH glycine washes. The cells were then washed once with PBS and the cells lysed to release internalized phage. The phage titer was then determined for each of the glycine washes and for the lysed cell fraction by infection of E. coli TG1.

Phage specificity	1st glycine wash	2nd glycine wash	lysed cell fraction
anti-Botulinum	6.0×10^5	1.0×10^5	6.0×10^5
Anti-ErbB2 (C6.5 scFv)	1.2×10^7	5.2×10^6	6.8×10^7
Anti-ErbB2 (C6.5 diabody)	1.8×10^7	2.8×10^6	1.7×10^7

Figure 5. Titer of cell surface bound phage and internalized phage. Graphic display of results from Table 4.

Legend:
☐ 1st glycine wash
☐ 2nd glycine wash
■ lysed cell fraction

Y-axis: Titer (1.0E+05, 5.0E+07, 1.0E+08, 1.5E+08, 2.0E+08)
X-axis: Irrelevant, scFv-C65, diab-C65

Significance: Taken together, the results indicate that: 1) phage binding cell surface receptors can be taken up by cells and the infectious phage recovered from the cytoplasm. The amount of uptake is significantly greater than uptake of non-binding phage, and the 100 to 200 fold difference is well within the range that would allow enrichment from a library. What is unknown from the results is whether the phage antibodies are mediating receptor mediated internalization or whether they are merely taken up after binding by membrane turnover.

2.3B   Selection and characterization of internalizing antibodies from a phage antibody library

The results described in section 2.3A encouraged us to attempt selection of the phage antibody library described in section 2.2 to identify new phage antibodies that were internalized. Phage antibodies were rescued from the library and selected on SKBR3 cells. For selection, phage were incubated with cells at 37°C, non-binding phage removed by washing cells with PBS and phage bound to cell surface antigens removed by sequential washes with low pH glycine. Cells were then lysed to release internalized phage and the lysate used to infect E. coli TG1 to prepare phage for the next round of selection. Three rounds of selection were performed. One hundred clones from each round of selection were analyzed for binding to SKBR3 cells and to ErbB2 extracellular domain by ELISA. We hypothesized that we were likely to obtain binders to ErbB2 since SKBR3 cells are known to express high levels and ErbB2 is a receptor which is known to be internalized. After each round of selection, the titer of phage recovered from the cytoplasm increased (Table 5). After the third round, 45% of the clones were positive SKBR3 cell binding and 17% bound ErbB2 (Table 5).

Table 5. Results of selection of a phage antibody library for internalization. For each round of selection, the titer of phage in lysed cells, number of cells lysed and number of phage per cell is indicated. After the third round, individual clones were analyzed for binding to SKBR3 cells by ELISA and to ErbB2 ECD by ELISA.

Round of selection	# of phage in cell lysate	# of cells lysed	# of phage/cell	% SKBR3 binders	% ErbB2 binders
1	3.5×10^4	2.8×10^6	0.013	ND	ND
2	1.2×10^5	2.8×10^6	0.038	ND	ND
3	7.5×10^6	2.8×10^6	3.75	45%	17%

To estimate the number of unique binders, the scFv gene from ELISA positive clones was PCR amplified and fingerprinted by digestion with BstN1. Two unique restriction patterns were identified. The scFv genes were sequenced and 2 unique ErbB2 binding scFv identified. Similar analysis of SKBR3 ELISA positive clones that did not bind ErbB2 identified an additional 11 unique scFv.

To verify that phage antibodies were specific for SKBR3 cells, phage were prepared from each unique clone and analyzed for binding to SKBR3 cells (high ErbB2 expression) as well as 2 other epithelial tumor cell lines (SK-OV-3, moderate ErbB2 expression and MCF7, low ErbB2 expression) and a normal breast cell line(HST578). Each unique clone specifically stained tumor cell lines but not the normal breast cell line. Representative results are shown in figures 6 and 7. In figure 6, SKBR3 and MCF7 cells are incubated with phage antibodies C6.5 (positive control), 3TF5 and 3GH7. The latter two clones were isolated from the library, with 3TF5 binding ErbB2 and the antigen bound by 3GH7 unknown. All 3 phage antibodies intensely stain SKBR3 cells (the selecting cell line and high ErbB2 expresser. C6.5 phage weakly stain MCF7 cells (low ErbB2 expressor. The anti-ErbB2 clone 3TF5 from the library stains MCF7 cells much more intensely then C6.5, as does 3GH7.

SKBR3 cells	MCF7 cells
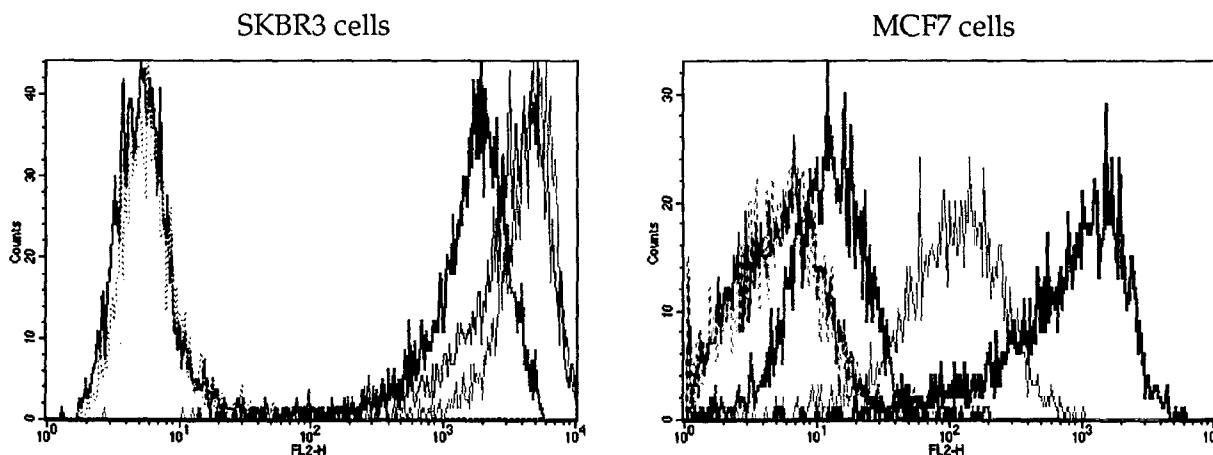
Figure 6. Staining of SKBR3 and MCF7 cells with phage antibodies as analyzed by FACS. 5.0×10^{12} phage/ml were incubated with cells. Binding was detected using biotinylated sheep anti-M13 and streptavidin-Texas red. Gray = 1° and 2° antibodies only, pink = anti-botulinum phage, green = C6.5-phage, orange = 3TF5 phage (anti-ErbB2), blue = 3GH7 phage.

In figure 7, SKBR3, SK-OV-3, MCF7 and HST578 cells were studied using native purified scFv 3TF5 and 3GH7. For these studies, the scFv genes were subcloned into a vector which fuses a hexahistidine tag to the scFv C-terminus. scFv was then expressed, harvested from the bacterial periplasm and purified by immobilized metal affinity chromatography. As in figure 6, the two scFv intensely stain SKBR3 cells, and do not stain the normal breast cell line HST578. There is minimal staining of the low ErbB2 expressing cell line MCF7 and intermediate staining of SK-OV-3 cells (moderate ErbB2 expresser). In general, the intensity of staining is less than seen

with phage. This is to be expected since the secondary antibody for phage staining recognizes the major coat protein (2500 copies/phage) resulting in tremendous signal amplification.

SKBR3 cells   MCF7 cells

HST578 cells   SK-OV-3 cells
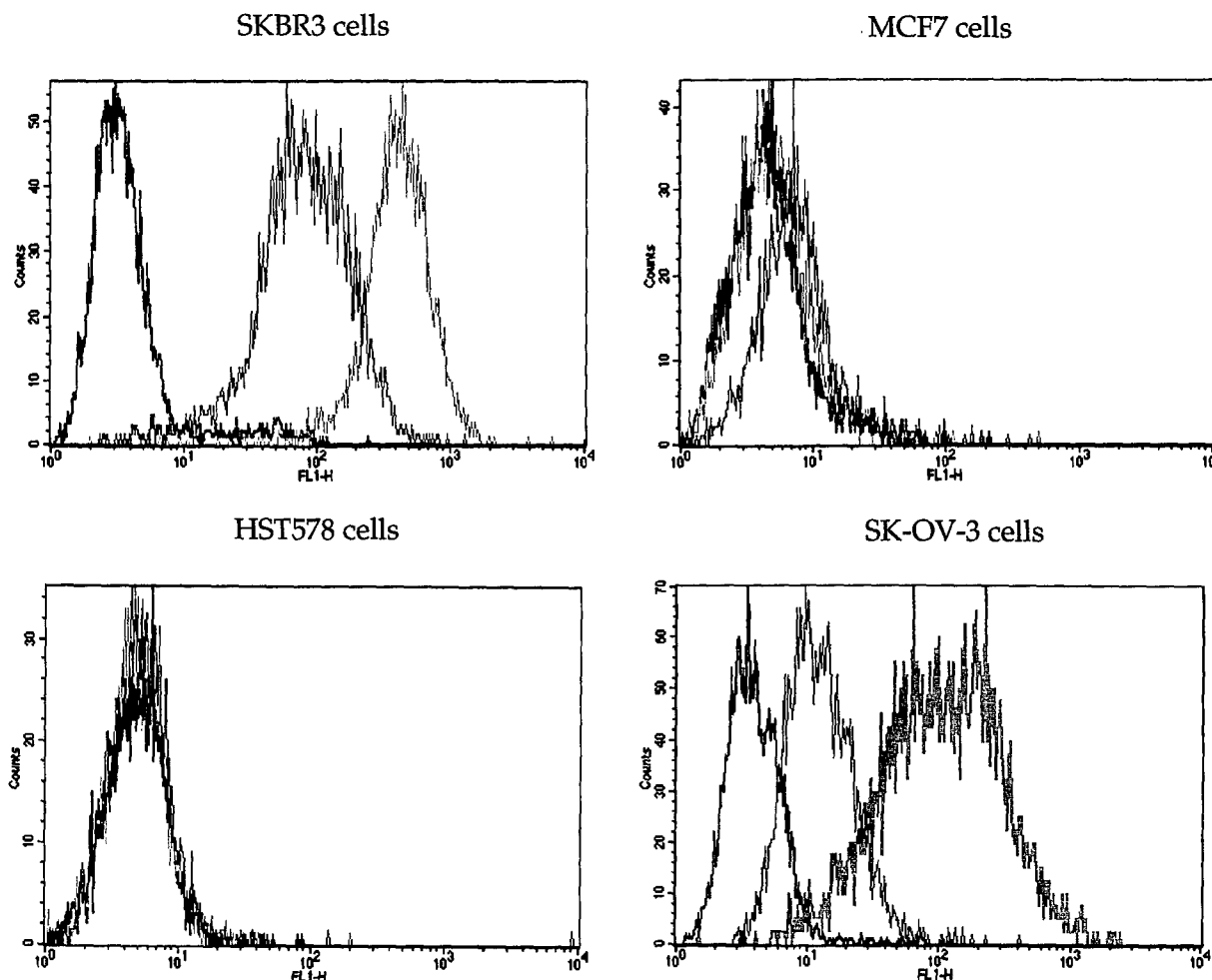
Figure 7. Staining of SKBR3, SK-OV-3, MCF7 and HST578 cells with scFv as analyzed by FACS. Cells were incubated with purified scFv (0.5 uM) and binding detected using mAb 9E10 (recognizes C-terminal myc epitope tag) and FITC-goat anti-mouse Fc. Gray = 1° and 2° antibodies only, orange = 3TF5 scFv (anti-ErbB2), blue = 3GH7 scFv.

2.3C   Selected antibodies are efficiently internalized

The anti-ErbB2 phage antibody 3TF5 was studied further to determine if it was indeed internalized. This antibody was selected for initial study since its internalization could be compared to ErbB2 binding C6.5. 5.0 x10^{11} 3TF5 or C6.5 phage were incubated with SKBR3 cells at 37°C or at 4°C. After washing with PBS, 3TF5 phage stained cells more intensely than C6.5 phage (Figure 8). After washing with low pH glycine, confocal microscopy revealed that 3TF5 phage were internalized by greater than 95% of cells, while C6.5 was internalized by only a few percent of cells (Figure 8). Incubation of either antibody at 4°C led to no internalization (Figure 8).

The native purified 3TF5 scFv was similarly analyzed and was also efficiently internalized by SKBR3 cells (Figure 9). It should be noted that the native 3TF5 scFv existed only as a monomer with no appreciable dimerization or aggregation as determined by gel filtration.

PBS Wash   Glycine Wash   Glycine wash (4°C incubation)
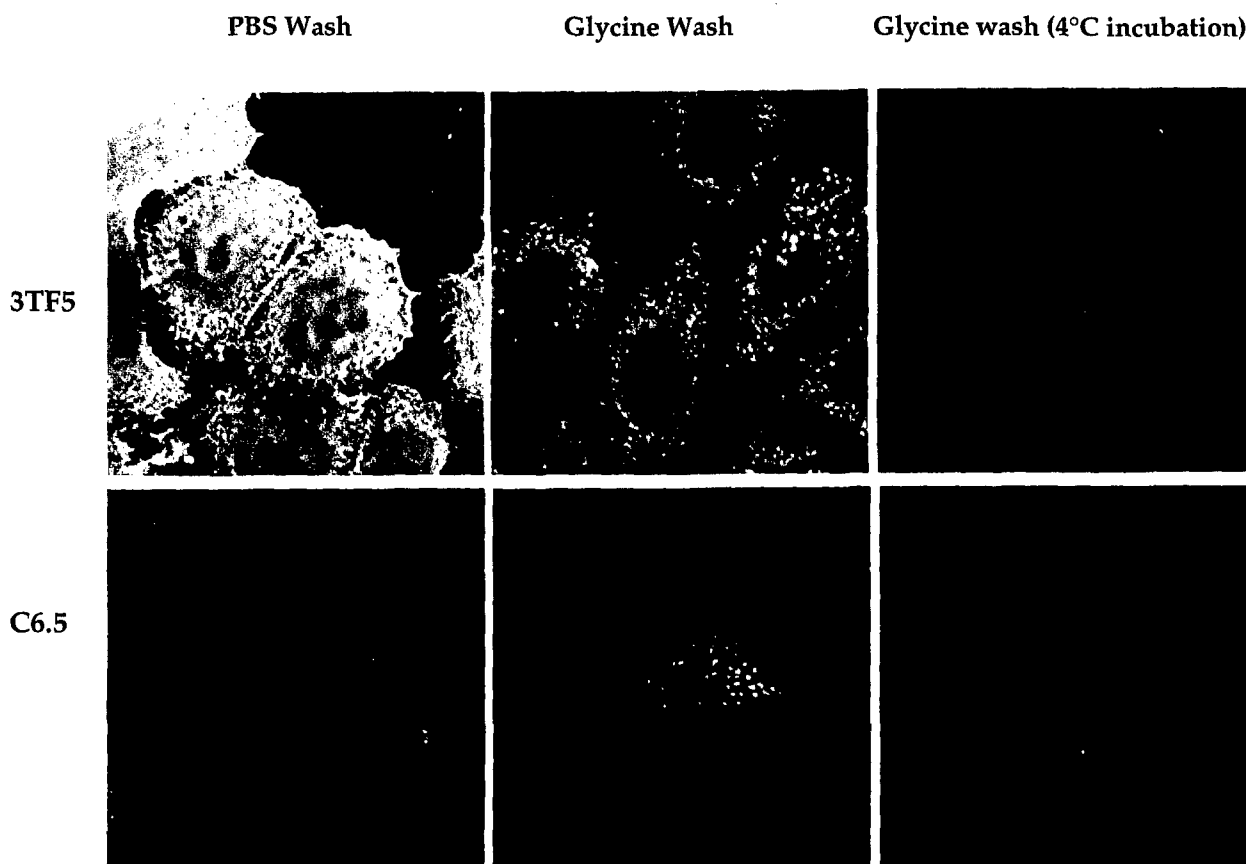
Figure 8. Internalization of phage as determined by immunofluorescence and confocal microscopy. SKBR3 cells were incubated with C6.5 phage (bottom panels) or 3TF5 phage (top panels) at 37°C first two columns) or at 4°C (far right column). Cells were washed with PBS (tleft column) to remove unbound phage antibody, followed by low pH glycine (middle and far left columns) to remove surface bound phage antibody. Phage antibodies were visualized using biotinylated anti-M13 antibody and streptavidin Texas red.

Incubate at 4°C   Incubate at 37°C
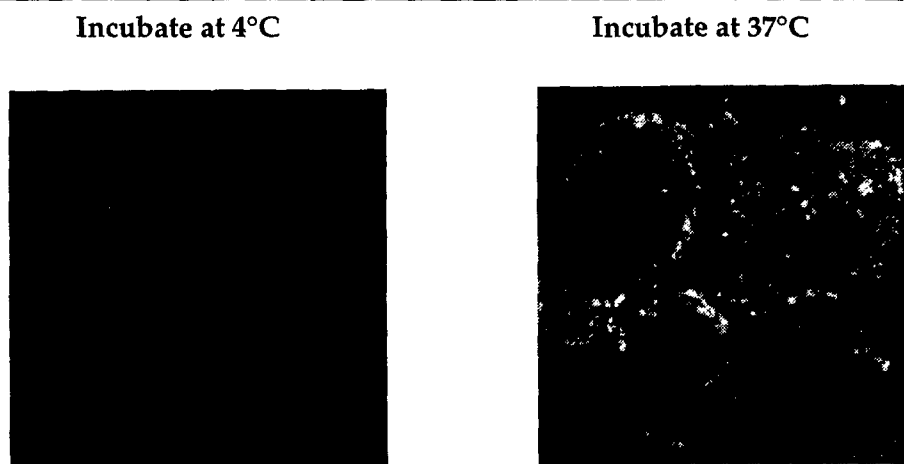
Figure 9. Internalization of native and 3TF5 scFv by SKBR3 cells. Cells were incubated with purified native 3TF5 scFv at either 37°C (right panel) or 4°C (left panel). Unbound scFv was removed by washing with PBS and surface bound scFv removed by washing with glycine.

Significance: Using a model system, we have demonstrated that phage antibodies can be internalized by cells and recovered from the cytoplasm. Phage that bind an internalizing cell surface receptor can be enriched more than 100 fold over non-binding phage. This level of enrichment is greater than that achieved by selecting on the cell surface. We have applied this approach to library selection and isolated phage antibodies that bind and are internalized by SKBR-3 cells. Several of these antibodies bind to ErbB2, but are more efficiently internalized than antibodies such as C6.5 that were generated by selecting on pure antigen. Many other antibodies have been isolated that bind specifically to SKBR-3 and other breast tumor cell lines and are efficiently internalized. These antibodies should prove useful for tumor targeting and for identifying potentially novel internalizing tumor cell receptors.

2.4.   Technical objective 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.

Year 1 to 3

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

To answer these questions, we studied the human scFv C6.5, which binds the extracellular domain (ECD) of the tumor antigen ErbB-2 (32) with a K_d of 1.6×10^{-8} M and k_{off} of 6.3×10^{-3} s^{-1} (26). Isolation and characterization of C6.5 is described briefly below and in detail in Schier et al., appendix 1. The isolation and initial characterization of C6.5 was partially supported by this grant, as well as by a subcontract to the Marks lab by National Cooperative Drug Discovery Group Group Award U01 CA 51880. Despite excellent tumor:normal tissue ratios in vivo, quantitative delivery of C6.5 was not adequate to cure tumors in animals using radioimmunotherapy (26). To improve the quantitative delivery of antibody to tumor, the affinity of C6.5 was increased. First, techniques were developed that allowed selection of phage antibodies on the basis of affinity, rather than differential growth in E. coli or host strain toxicity (27-29). Next, we determined which locations in the scFv gene to mutate to achieve the greatest increments in affinity (27, 28, 30). Random mutagenesis did not yield as great an increment in affinity as site directed mutagenesis of the complementarity determining regions (CDRs) that contain the amino acids which contact antigen. Results from diversifying the CDRs indicated that: 1) the greatest increment in affinity was achieved by mutating the CDRs located in the center of the binding pocket (V_L and V_H CDR3); 2) half of the CDR residues have a structural role in the scFv and when mutated return as wild-type; and 3) these structural residues can be identified prior to library construction by modeling on a homologous atomic crystal structure. These observations led to development of a generic strategy for increasing antibody affinity where mutations are randomly introduced sequentially into V_H and V_L CDR3, with conservation of residues postulated to have a structural role by homology modeling (30). Using this approach, the affinity of C6.5 was increased 1200 fold to a K_d of 1.3×10^{-11} M (30).

Biodistribution studies revealed a close correlation between affinity and the percent injected dose of scFv/gram of tumor (%ID/g) at 24 hours (31). The greatest degree of tumor retention was observed with ^{125}I-C6ML3-9 (1.42 %ID/g, K_d = 1.0 x 10^{-9} M). Significantly less tumor retention was achieved with ^{125}I-C6.5 (0.80 %ID/g, K_d = 1.6 x 10^{-8}) and C6G98A (0.19 %ID/g, K_d = 3.2 x 10^{-7} M). The tumor:normal organ ratios also reflected the differences in affinity, e. g. tumor:blood ratios of 17.2, 13.3, 3.5 and 2.6, and tumor to liver ratios of 26.2, 19.8, 4.0 and 3.1 for C6ML3-9, C6.5 and C6G98A respectively at 24 hours. Studies of the higher affinity scFv are pending. The results demonstrate our ability to increase antibody affinity to values not achievable from hybridoma technology and confirm the importance of affinity in tumor targeting

2.4A.   Preclinical development of C6.5 based breast cancer therapies

Two approaches have been collaboratively pursued to develop C6.5 based breast cancer therapies. In collaboration with Lou Weiner's group at Fox Chase Cancer Center, C6.5 based molecules are being engineered for radioimmunotherapy. To increase quantitative tumor delivery and retention of antibody fragment, dimeric scFv 'diabodies' were created by shortening the linker between the V_H and V_L domains from 15 to 5 amino acids. Consequently, pairing occurs between complementary domains of two different chains, creating a stable noncovalently bound dimer with two binding sites. In vitro, diabodies produced from the V-genes of C6.5 have a significantly higher apparent affinity and longer retention on the surface of SK-OV-3 cells compared to C6.5 scFv ($T_{1/2}$ > 5 hr vs. 5 min) (32). Biodistribution studies of C6.5 diabody revealed 6.5 %ID/g tumor at 24 hours compared to only 1 %ID/g for C6.5 scFv. When diabody retentions were examined over 72 hours and cumulative area under the curve (AUC) values determined, the resulting tumor:organ AUC ratios were greater than reported for other monovalent or divalent scFv molecules. The therapeutic potential of these molecules is being examined in radioimmunotherapy studies in nude mice. Since in vivo characterization of c6.5 based molecules was not formally one of the technical objectives, we are continuing to use the affinity mutants of C6.5 and C6.5 based diabodies to study the relationship between antibody affinity, size and valency and specific tumor targeting as part of NIH R01 1 CA65559-01A1.

In collaboration with Dr. Chris Benz's group at UCSF and the Bay Area Breast Cancer SPORE, C6.5 based molecules are being used to target doxorubicin containing stealth liposomes to ErbB2 expressing breast cancers (34). To facilitate chemical coupling of the scFv to liposomes, the C6.5 gene was subcloned into an E. coli expression vector resulting in addition of a free cysteine residue at the C-terminus of the scFv. Purified C6.5cys scFv was conjugated to liposomes and in vitro uptake determined using SKBR3 cells. Total uptake was 3.4 mmol phospholipid/10^6 cells at 6 hour, with 70% of the uptake internalized. The uptake is comparable to that achieved using the 4D5 anti-HER2 Fab' from Genentech. There was no uptake of unconjugated liposomes. The results indicate that C6.5 binds to a ErbB2 epitope that results in internalization at a rate comparable to the best internalizing antibody produced from hybridomas (4D5). In vivo therapy studies in scid mice indicated that C6.5 targeted liposomes caused a greater degree of tumor regression and a higher cure rate than untargeted liposomes or a combination of untargeted liposomes and systemic 4D5 antibody. At a December meeting, the National Cancer Institute Decision Network voted to support further development of C6.5 targeted liposomes for breast cancer therapy at the 2A level. This means support for development of processes to increase antibody expression and scale up of targeted liposome production for toxicology studies in anticipation of GMP manufacturing for a clinical trial. Engineering of C6.5 based molecules for the liposome project was funded by the Bay Area Breast Cancer SPORE, but I thought the reviewers would be interested to know how an antibody developed under DOD BCRP funding was proceeding towards clinical trials.

Significance: Phage antibody techniques and protocols have been developed which permit engineering of ultra high affinity human antibodies to any antigen. The technology has been applied to produce ErbB2 binding scFv with novel properties. Compared to ErbB2 antibodies which are currently in clinical trials for cancer therapy, the antibodies are entirely human in

sequence and bind with higher affinity ($K_d = 1.3 \times 10^{-11}$ M). These unique features have led the University of California to file a patent titled "High affinity human antibodies to novel tumor antigens". We are currently pursuing two separate approaches to develop these molecules for therapy of ErbB2 expressing breast cancers. In addition, five Biotechnology/Pharmaceutical Companies are pursuing licenses to this technology as a means of targeting additional therapeutic agents to ErbB2 expressing cancers.

3.   Conclusions

1. A large (7.0 x 10^9 member) phage antibody library has been created which can provide panels of human antibodies to purified antigens with affinities comparable to the affinities of antibodies produced by murine immunization.

2. We have demonstrated that phage antibodies binding cell surface receptors can be can be internalized by cells and recovered in an infectious state from within the cell. Methodologies were developed which permit enrichment of internalizing phage antibodies over non-internalizing antibodies more than 100 fold.

3. We have applied these methodologies to select new scFv antibodies that bind to internalizing receptors on SKBR-3 cells. Several of these antibodies bind to ErbB2, but are internalized more efficiently than C6.5 based scFv. Many more antibodies bind to unknown internalizing receptors. All of these scFv bind specifically to SKBR-3 cells or related tumor cell lines.

4. The results indicate that this selection approach is a powerful approach to generate antibodies that can distinguish one cell type (malignant) from another (non-malignant). Moreover, we have demonstrated that it is not only possible to select for binding, but to select for function (internalization). In the near term, we will further characterize the antibodies isolated with respect to specificity, and in the case of ErbB2 binding scFv, affinity. In the longer term we will use these reagents to: 1) study the effect of affinity and valency on the rate of internalization; and 2) identify the antigens bound using immunoprecipitation. It is likely that the results will lead to the identification of novel internalizing tumor cell surface receptors which will be useful therapeutic targets. If this approach proves useful, we plan on applying it to primary tumor cells and DCIS. We also intend to evaluate 3TF5 (ErbB2 binding scFv which is internalized faster than C6.5) for liposome targeting. It is possible that it will be more effective than C6.5

5. Methodologies have been developed for increasing antibody affinity in vitro to values not previously achieved in vivo. We have applied these methodologies to generate novel ErbB2 binding scFv. Using funding from other sources, we are continuing to develop these molecules and are moving them towards the clinic for study as breast cancer therapeutics.

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Appendix 1

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

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Selection of higher affinity mutant phage antibodies has proven less than straightforward due to sequence dependent differences in phage antibody expression, toxicity to Escherichia coli, and difficulty in eluting the highest affinity phage. These differences lead to selection for increased levels of expression or decreased toxicity rather than for higher affinity. In this work, we demonstrate how surface plasmon resonance as employed in the BIAcore can be used to increase the efficiency of phage antibody selections, yielding greater increments in affinity from a single library. A mutant phage antibody library was created by randomizing nine amino acids located in the V_L CDR3 of C6.5, a human scFv which binds the tumor antigen c-erbB-2 with a K_d of 1.6×10^{-8} M. The library was subjected to five rounds of selection in solution using decreasing concentrations of biotinylated c-erbB-2. After each round of selection, polyclonal phage were prepared and the rate of binding to c-erbB-2 determined in a BIAcore under mass transport limited conditions. Determination of the rate of binding permitted calculation of the concentration, and hence percent, of binding phage present. Results were used to select the antigen concentration for the next round of selection. To determine the optimal eluent, polyclonal phage was injected in a BIAcore and eluted using one of five different solutions (10 mM HCl, 50 mM HCl, 100 mM HCl, 100 mM triethylamine, 2.6 M $MgCl_2$). Differences were observed in eluent efficacy, which was reflected in significant differences in the affinities of phage antibodies isolated from the library after a round of selection using the different eluents. Use of the BIAcore to determine the optimal eluent and guide the antigen concentration used for selection yielded a C6.5 mutant with a 16 fold reduction in K_d ($K_d = 1.0 \times 10^{-9}$ M). This represents at least a twofold greater increment in affinity than previously obtained from a single library of phage antibodies which bind antigens. [Hum Antibod Hybridomas 1996; 7: 97–105]

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

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[1]. The first two limitations have been largely overcome by the display of natural[2] and synthetic antibody variable region gene repertoires[3] on the surface of phage[4,5]. Human antibody fragments can be recovered from these libraries against virtually any antigen[2,6-9] with affinities for protein antigens ranging from 10^{-6} M to 10^{-8} M. Affinity of these primary isolates can be increased by creating mutant phage antibody libraries and selecting higher affinity antibodies[10-14].

Efficient selection of higher affinity mutant phage antibodies has proven less than straightforward due to

sequence dependent differences in phage antibody expression and in toxicity to *E. coli*. These differences can lead to selection for increased expression levels, or decreased toxicity, rather than for higher affinity. In the case of single-chain Fv (scFv) phage antibodies, selection is also complicated by the tendency of some scFv to dimerize. Dimeric scFv exhibit increased apparent affinity due to avidity and are preferentially enriched over monomeric scFv when selections are performed on antigen immobilized on a solid phase[14]. Thus selections must be carefully designed to ensure enrichment based on affinity, rather than expression level, toxicity to *E. coli*, or avidity. It has been previously shown that optimal selection of higher affinity scFv phage antibodies occurs when selections are performed in solution on biotinylated antigen with subsequent capture on streptavidin-coated magnetic beads[10-14]. For the initial round of selection, an antigen concentration greater than the K_d of the wild type scFv is used in order to capture

rare, or poorly expressed, phage antibodies. In subsequent rounds, the antigen concentration is reduced to significantly less than the desired K_d[14]. Use of too high an antigen concentration results in failure to sort on the basis of affinity, while use of too low antigen concentration results in loss of binding phage and subsequent overgrowth of deletion mutants[14]. The optimal antigen concentration cannot be predicted *a priori*, due to variability in phage antibody expression levels and uncertainty regarding the highest affinities present in the mutant phage antibody library. For selection of the highest affinity antibodies, it is also necessary to ensure that all specifically bound phage are eluted. Solutions used for elution include competition with soluble antigen[10,15,16], 100 mM triethylamine (triethylamine (TEA)[2,6,7,10,11,16], glycine, pH 2.2[17–20], 100 mM NaOAc, pH 2.8 containing 500 mM NaCl[21], or 76 mM citric acid, pH 2.8[22]. Alternatively, magnetic beads with bound phage can be added directly to *E. coli*[23].

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

Materials and methods

Library construction and phage preparation

A mutant phage antibody library was constructed based on the sequence of C6.5, a human scFv isolated from a nonimmune phage antibody library which binds the tumor antigen c-erbB-2 with a $K_d = 1.6 \times 10^{-8}$ M[24]. This library is described in detail in a separate publication[38]. The mutant phage antibody library C6VLCDR3 was constructed by partially randomizing nine amino acids (residues 89-95b, Kabat numbering[30]) located in the V_L CDR3 of C6.5. The ratio of nucleotides was chosen so that the frequency of wild type amino acid was 49% at each position randomized. The mutant C6.5 scFv gene repertoire was digested with SfiI and NotI and ligated into the phagemid vector pCANTAB5E (Pharmacia) digested with SfiI and NotI. After transformation, a library of 1.0×10^7

clones was obtained. For selection, phage were prepared as previously described[14].

Selection of the phage antibody library

The C6VLCDR3 library was subjected to five rounds of selection in solution on biotinylated c-erbB-2 ECD, as previously described[14], but with some modifications. After capture of phage, streptavidin-coated paramagnetic beads (Dynal) were washed a total of ten times (3 × phosphate buffered saline (25 mM NaH_2PO_4, 125 mM NaCl, pH 7.0, PBS) containing 0.05% Tween 20 (TPBS), 2 × TPBS containing 2% skimmed milk powder (MTPBS), 2 × PBS, 1 × PBS containing 2% skimmed milk powder (MPBS), and 2 × PBS) using a Dynal magnetic particle concentrator. The Dynabeads were resuspended in 1 ml PBS, and one third was used to infect 10 ml log phase *E. coli* TGl[31] which were plated on TYE plates containing 100 μg ml^{-1} ampicillin and 1% glucose (TYE-AMP-GLU)[32].

For determination of the effect of eluent, the fourth round of selection was repeated, exactly as described above, except that after washing, bound phage were eluted by adding 100 μl of one of seven eluents: 1. 100 mM HCl pH 1.0; 2. 50 mM HCl pH 1.3; 3. 10 mM HCl pH 2.0; 4. 2.6 M $MgCl_2$ 5. 100 mM TEA; 6. 1 μ c-erbB-2 ECD; or 7. no elution (magnetic beads resuspended in 1 ml of PBS). After five minutes incubation with eluent (15 min for the incubation with 1 μM c-erbB-2 ECD), the supernatant was transferred to a new tube and the mixture neutralized by the addition of 1.5 ml of 1 M Tris HCl pH 7.4. 500 μl of the elution mixture was used to infect 10 ml log phase *E. coli* TG1[31] which were plated on TYE-AMP-GLU plates.

BIAcore and ELISA screening

Phage ELISA were performed to determine the percentage of antigen binding clones. 96 single clones were picked from the unselected library and after each round of each selection, resuspended in 200 μl 2 × TY-AMP-GLU, and grown over night at 37°C in a 96-well microtitre plate (Corning). Aliquots of bacteria were transferred to a new 96-well microtitre plate containing 100 μl 2 × TY-AMP-0.1% glucose and grown to an OD_{600} of approximately 0.7. 50 μl of VCS-M13 helper phage (Stratagene) (2.5 × 10^8 pfu/ml) were added to each well, and the wells incubated for 1h at 37°C without shaking. 50 μl of 2 × TY-AMP containing 100 μg ml^{-1} kanamycin were added per well, and the bacteria grown overnight at 37°C. Bacteria were spun down at 2000 rpm in a Beckman GS-65 centrifuge and supernatant containing phage used for ELISA.

For phage ELISA, Immunolon 4 microtiter plates (Dynatech) were coated with 50 μl ImmunoPure avidin (Pierce; 10 μg ml^{-1} in PBS) overnight at 4°C, blocked with 1% bovine serum albumin in PBS for an hour at 37°C, incubated with 50 μl biotinylated c-erbB-2 ECD (5 μg ml^{-1} in PBS) for 30 min at 20°C, followed by an incubation with 50 μl *E. coli* supernatant containing phage for one hour at 20°C. Binding of scFv phage to the antigen was detected with a peroxidase-conjugated anti-M13 monoclonal antibody (Pharmacia) using ABTS as substrate.

The reaction was stopped after 30 min with NaF (3.2 mg ml^{-1}) and the A$_{405nm}$ measured.

Screening of scFv by k$_{off}$ was performed using real-time biospecific interaction analysis based on SPR in a BIAcore (Pharmacia) as described by Schier *et al.*[14]. Ten ml cultures of *E. coli* TG1 containing the appropriate phagemid was grown and expression of scFv induced with isopropylβ–D–thiogalactopyranoside (IPTG)[33]. Cultures were grown overnight at 25°C, scFv harvested from the periplasm[34], and the periplasmic fraction dialyzed for 24 h against HBS. In a BIAcore flow cell, approximately 1400 RU of c-erbB-2 ECD (25 μg ml^{-1}) in 10 mM acetate buffer pH 4.5 were coupled to a CM5 sensor chip using NHS/EDC amine coupling chemistry[35]. Dissociation of undiluted periplasmic fraction of *E. coli* containing scFv was measured under a constant flow of 5 μl min^{-1}. An apparent k$_{off}$ was determined from the dissociation part of the sensorgram for each scFv analyzed[36].

Subcloning, expression and purification of scFv

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

Measurement of affinity and binding kinetics

The K$_d$ of scFv were determined using SPR in a BIAcore. In a BIAcore flow cell, approximately 1400 RU of c-erbB-2 ECD (90 kDa; 25 μg ml^{-1} in 10 mM sodium acetate pH 4.5) were coupled to a CM5 sensor chip[35]. Association (k$_{on}$) and k$_{off}$ were measured under continuous flow of 5 μl min^{-1} using a concentration range of scFv from 50 to 800 nM. k$_{on}$ was determined from a plot of (ln(dR/dt))/t vs concentration[36]. To verify that differences in k$_{on}$ were not due to differences in immunoreactivity, the relative concentration of functional scFv was determined using SPR in a BIAcore and was within 5% of the concentration determined by A$_{280}$. k$_{off}$ was determined from the first 1.5 min of the dissociation part of the sensorgram at the highest concentration of scFv analyzed[36]. To exclude rebinding, k$_{off}$ was determined in the presence and absence of 5.0 × 10^{-7} M c-erbB-2 ECD as previously described[14]. No significant differences in k$_{off}$ were observed between samples analyzed in the presence and absence of c-erbB-2 ECD.

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

The efficacy of different elution solutions in removing polyclonal phage from c-erbB-2 ECD was determined using SPR in a BIAcore (Pharmacia). CM5 sensor chip flow cells were coated with 1800 RU of c-erbB-2 ECD (50 μg ml^{-1} in 10 mM sodium acetate pH 4.5). Polyclonal phage were prepared[14] after the third round of selection and resuspended in HBS. 30 μl of phage (5 × 10^{12} cfu ml^{-1} were injected over the flow cell surface using HBS as running buffer and a flow rate of 5 μl min^{-1}. 2.5 min into the dissociation period, 5 μl of one of six eluents (HBS, 2.6 M MgCl$_2$, 100 mM TEA, 10 mM HCl, 50 mM HCl, or 100 mM HCl) was injected over the flow cell surface at a rate of 5 μl min^{-1}, followed by a wash step. The amount of phage bound was determined 15 sec after the end of the association phase and six minutes later at the end of the wash step. The differences between these two points was used to calculate the percent of phage still bound after elution.

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

A standard curve was constructed using monoclonal C6.5 scFv phage[25]. Phage were prepared and titred (cfu ml^{-1}) on *E. coli* TG1[14]. In a BIAcore, 1800 RU of c-erbB-2 ECD was coupled to a CM5 sensor chip using NHS-EDC chemistry. Thirty ml aliquots of C6.5 phage (1.0 × 10^{11} to 1.0 × 10^{13} cfu ml^{-1}) were injected over the flow cell surface using a running buffer of PBS containing 0.05% P20 and a flow rate of 5 μl min^{-1}. Binding rates were calculated in RU/min from the association portion of each sensorgram. The amount of phage bound (RU) was also determined using a reference point taken 15 sec after the end of the association phase. Two standard curves were constructed, one plotting the log of the phage titre vs the log of the binding rate, and one plotting the log of the phage titre vs the log of the RU phage bound (*Figure 1*).

To determine the concentration of binding phage in the polyclonal phage mixture, phage were prepared after each round of selection and the titre determined[14]. Thirty μl aliquots of phage (3.0 to 8.0 × 10^{12} cfu ml^{-1}) were injected over the flow cell surface, the binding rate and RU bound measured, and the concentration (cfu ml^{-1}) of c-erbB-2 binding phage determined from the standard curves. The percent binding phage was calculated as the ratio of the concentration of the binding phage (cfu ml^{-1})/total phage titre determined by infection of *E. coli* (cfu ml^{-1}).

Results

Monitoring phage antibody selection using surface plasmon resonance in a BIAcore

A technique was developed to measure the concentration of antigen binding phage present in a polyclonal phage mixture. To construct a standard curve relating the concentration of binding phage to BIAcore response, phage were prepared from the anti-c-erbB-2 monoclonal antibody

C6.5 [24]. The phage concentration (colony forming units (cfu) ml^{-1}) was determined by titration on *E. coli* TG1. The response signal (resonance units; RU) and rate (RU min^{-1}) of binding of serial dilutions of C6.5 scFv phage to c-erbB-2 extracellular domain (c-erbB-2 ECD) were determined in a BIAcore under mass transport limited conditions[25]. A plot of the log of the phage concentration versus either the binding rate, or the amount of phage bound yielded linear standard curves *(Figure 1)*.

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

During the first four rounds of selection, the titre of eluted phage decreased as the antigen concentration used for selection decreased *(Table 1)*. The concentration and percentage of binding phage as determined by SPR, however, increased each round *(Table 1)*. The percentage of individual colonies expressing scFv which bound c-erbB-2 ECD, as determined by ELISA, also increased each round, and the values correlated closely with the percent of binding phage determined by BIAcore *(Table 1)*. These results suggest successful antigen driven selection. This was confirmed by measuring the K$_d$ of native scFv expressed from 37 clones from the fourth round of selection (see *Table 4* and the next section). All scFv had a lower K$_d$ than the parental C6.5 scFv, with the best scFv having 16 fold decreased K$_d$. Further reduction of the antigen
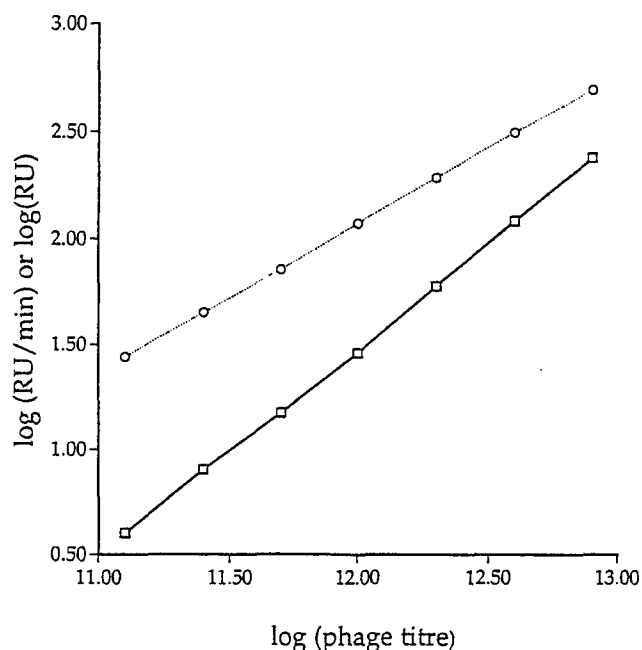
Figure 1: Correlation between phage binding in a BIAcore and the phage titre. Monoclonal C6.5 scFv (1.0 × 10^{11} to 1.0 × 10^{13} cfu ml^{-1}) were injected over a c-erbB-2 ECD coupled sensor chip in a BIAcore and the rate of binding (RU min^{-1}) and amount (RU) of phage bound determined. Two standard curves were constructed, one plotting the log of the phage concentration (cfu m^{-1}) vs the log of the binding rate (RU min^{-1}), □; and one plotting the log of the phage concentration (cfu/ml) vs the log of the amount of phage bound (RU), O. These standard curves were used to calculate the titre of binding phage in polyclonal mixtures of phage

concentration to 1 × 10^{-12} M in a fifth round of selection resulted in a large reduction of the BIAcore response, indicating loss of binding phage antibodies due to excessive stringency *(Table 1)*. Loss of phage binding in the BIAcore correlated with an absence of binding as determined by ELISA. Loss of binding was paradoxically associated with an 800 fold increase in the titre of eluted phage *(Table 1)*. PCR screening of 20 clones after the fifth round of selection indicated that most clones had lost part of the scFv gene.

Table 1: Results of selection of the C6VLCDR3 phage antibody library on c-erbB-2 ECD

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

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

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

To determine if differences existed in the ability of eluents to remove antigen bound phage, polyclonal phage were prepared after three rounds of selection of the C6VLCDR3 library and studied using SPR in a BIAcore. After an initial bulk refractive index change, binding of phage to immobilized c-erbB-2 ECD was observed, resulting in an average of 189 RU bound (*Table 2, Figure 2*). Phage were then allowed to either spontaneously dissociate from c-

erbB-2 ECD using hepes buffered saline (HBS) as running buffer, or were eluted with either 100 mM HCl, 50 mM HCl, 10 mM HCl, 2.6 M MgCl$_2$, or 100 mM TEA. Major differences were observed between eluents in their ability to remove bound phage (*Table 2, Figure 2*). The most effective solutions in removing bound phage antibodies were 100 mM HCl and 50 mM HCl, followed by 100 mM TEA. 2.6 M MgCl$_2$ (which removes 100% of wild type C6.5) and 10 mM HCl were only minimally more effective than the running buffer in removing bound phage.

Table 2: Effects of different eluents on removing bound phage from c-erbB-2 ECD as determined by surface plasmon resonance in a BIAcore

Eluent	RU phage bound before elution	RU phage bound after elution	% 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

Polyclonal anti-c-erbB-2 phage prepared after the third round of selection were injected over c-erbB-2 ECD coupled to a sensor chip in a BIAcore. After association, the amount (RU) of bound phage was determined, one of six eluents injected over the sensor chip surface, and the amount of phage that remained bound to c-erbB-2 determined. Major differences were observed in the efficacy of eluents in removing bound phage.
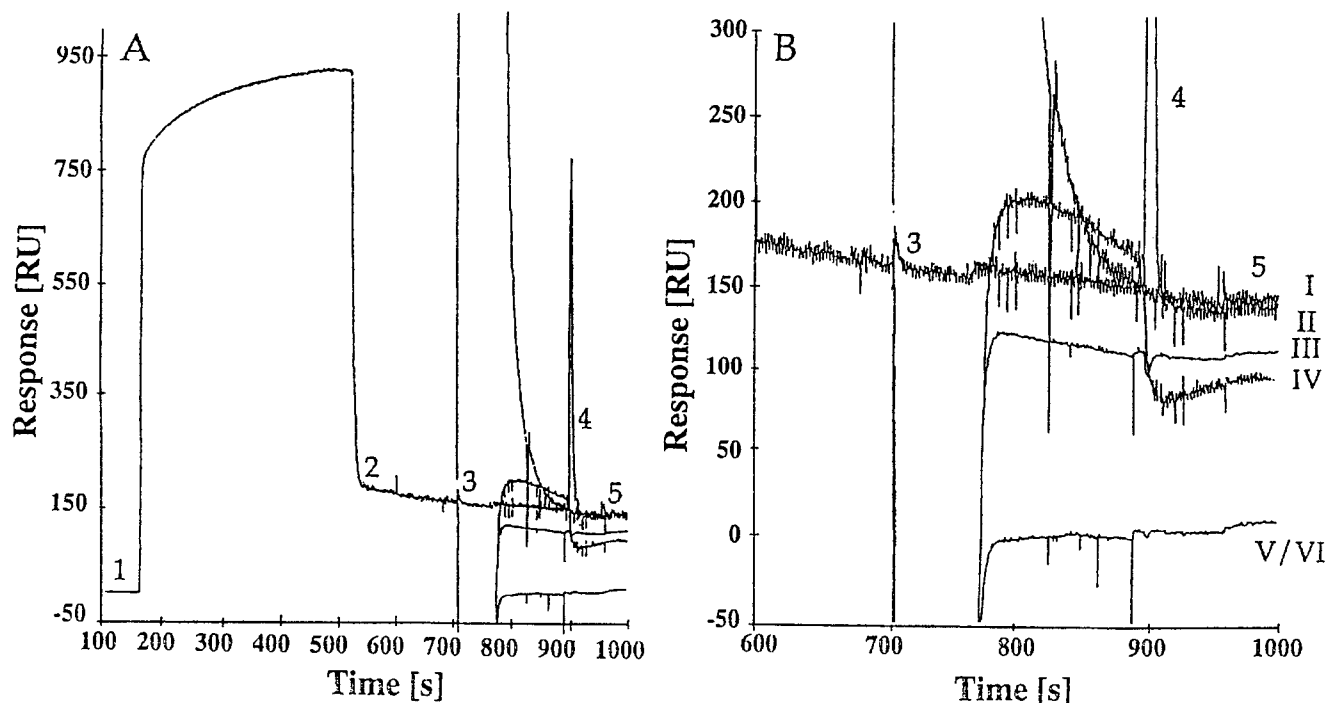
Figure 2: Effect of different eluents on removing bound phage from c-erbB-2 ECD. Polyclonal anti-c-erbB-2 phage were injected over a c-erbB-2 ECD coupled sensor chip in the BIAcore and the ability of six different eluents to remove bound phage was determined. **A:** overlay plot of the six sensorgrams generated from analysis of the six eluents: 1. baseline, beginning of association; 2. beginning of dissociation; difference between point 1 and 2 = amount of phage bound; 3. beginning of elution, differences in refractive index of eluents results in large positive or negative changes in RU, depending on the eluent used; 4. washing out the eluent from the flowcell; 5. amount of phage bound after elution. **B:** Enlargement of (A) between points 3 and 5. Significant differences exist in the amount of phage remaining bound after elution. (I : HBS; II : 2.6 M MgCl$_2$; III : 10 mM HCl; IV : 100 mM TEA; V : 50 mM HCl; VI : 100 mM HCl)

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Table 3: Effect of different eluents on the selection of higher affinity phage antibodies

Eluent	Titre of eluted phage	ELISA positive clones	K_d ($\times 10^{-9}$ M)	k_{on} ($\times 10^5$ s^{-1}M^{-1})	k_{off} ($\times 10^{-3}$ s^{-1})
No elution[a]	5.2×10^4	75/92	5.39 ± 0.73	4.64 ± 0.35	2.49 ± 0.41
1 μM c-erbB-2	6.0×10^4	82/92	5.99 ± 1.12	5.09 ± 0.27	2.58 ± 0.47
2.6 M MgCl$_2$	1.1×10^4	83/92	3.30 ± 0.45^c	5.05 ± 0.43	1.58 ± 0.14
100 mM TEA	1.2×10^4	89/92	2.65 ± 0.35^b	4.78 ± 0.39	1.27 ± 0.20
10 mM HCl	1.0×10^4	85/92	6.09 ± 1.29	5.72 ± 0.30	3.46 ± 0.80
50 mM HCl	1.0×10^4	90/92	2.60 ± 0.40^b	6.38 ± 1.02	1.54 ± 0.19
100 mM HCl	2.1×10^3	87/92	2.52 ± 0.46^b	5.99 ± 0.37	1.40 ± 0.20

Polyclonal phage was subjected to a fourth round of selection on c-erbB-2 ECD and the bound phage eluted with one of seven eluents (column 1). The titre of eluted phage (column 2) and the number of individual clones binding c-erbB-2 was determined by ELISA. scFv with the lowest k_{off} were identified by BIAcore screening, the scFv purified, and binding kinetics (k_{on} and k_{off}) determined by BIAcore and used to calculate the K_d. Significant differences in the K_d of selected scFv were observed.
[a], magnetic beads with bound phage added directly to *E. coli* culture; [b] $p < 0.05$ compared to no elution, 1 μM c-erbB-2, and 10 mM HCl; [c] $p < 0.05$ compared to 10 mM HCl.

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

Clone	V_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	A A W D D S L S G W V	16.0	4.0 ± 0.20	6.3 ± 0.06
No elution				
C6ML3-5(4)	- - - - - Y - - - - -	3.7	5.1 ± 0.34	1.9 ± 0.09
C6ML3-17	- S - - Y Y R - - - -	4.9	3.5 ± 0.22	1.7 ± 0.02
C6ML3-1	- - - - Y - - W - - -	6.1	3.3 ± 0.07	2.0 ± 0.15
C6ML3-22	- - - - A - - - - - -	8.3	4.3 ± 0.21	3.6 ± 0.02
C6ML3-26	- - - - - - R - - - -	8.3	6.0 ± 0.77	5.0 ± 0.04
1 μM c-erbB-2				
C6ML3-5(5)	- - - - Y - - - - - -	3.7	5.1 ± 0.34	1.9 ± 0.09
C6ML3-17	- S - - Y Y R - - - -	5.0	3.5 ± 0.22	1.7 ± 0.02
C6ML3-25(2)	- - - - N R H - - - -	7.4	5.9 ± 0.72	4.4 ± 0.03
2.6 M M$_g$Cl$_2$				
C6ML3-12	- - - - Y - R - - - -	1.6	4.5 ± 0.16	0.72 ± 0.02
C6ML3-15	- - - - R P - W - - -	2.2	5.9 ± 0.8	1.3 ± 0.02
C6ML3-7(2)	- - - - Y A V - - - -	2.6	6.5 ± 0.29	1.7 ± 0.09
C6ML3-5(2)	- - - - Y - - - - - -	3.7	5.1 ± 0.34	1.9 ± 0.09
C6ML3-16	- S - - Y - R - - - -	3.8	5.5 ± 0.12	2.1 ± 0.05
C6ML3-17	- S - - Y Y R - - - -	4.9	3.5 ± 0.22	1.7 ± 0.02
100 mM TEA				
C6ML3-19	- S - - R P - W - - -	1.5	6.6 ± 0.69	1.0 ± 0.02
C6ML3-12	- - - - Y - R - - - -	1.6	4.5 ± 0.16	0.72 ± 0.02
C6ML3-18	- S - - A - - W - - -	2.4	2.6 ± 0.08	0.62 ± 0.02
C6ML3-20	- - - E Q - - W - - -	3.0	4.7 ± 0.08	1.4 ± 0.02
C6ML3-5(3)	- - - - Y - - - - - -	3.7	5.1 ± 0.34	1.9 ± 0.09
10 mM HCl				
C6ML3-23	- S - - H - - W - - -	1.5	6.7 ± 0.41	1.0 ± 0.02
C6ML3-7	- - - - Y A V - - - -	2.6	6.5 ± 0.29	1.7 ± 0.09
C6ML3-5	- - - - Y - - - - - -	3.7	5.1 ± 0.34	1.9 ± 0.09
C6ML3-21	- - - - Y - Q - - - -	4.5	4.9 ± 0.01	2.2 ± 0.05
C6ML3-25	- - - - N R H - - - -	7.4	5.9 ± 0.72	4.4 ± 0.03
C6ML3-22	- - - - A - - - - - -	8.3	4.3 ± 0.21	3.6 ± 0.02
C6ML3-26	- - - - - - R - - - -	8.3	6.0 ± 0.77	5.0 ± 0.04
C6ML3-24	- - - - E Q I F - - -	12.4	6.4 ± 0.89	7.9 ± 0.04
50 mM HCl				
C6ML3-12(2)	- - - - Y - R - - - -	1.6	4.5 ± 0.16	0.72 ± 0.02
C6ML3-29	- - - - G T - W - - -	1.7	12.9 ± 1.03	2.2 ± 0.02
C6ML3-28	- S - - Y A - - - - -	2.5	6.8 ± 0.17	1.7 ± 0.02
C6ML3-7(2)	- - - - Y A V - - - -	2.6	6.5 ± 0.29	1.7 ± 0.09
C6ML3-6	- S - - Y - - - - - -	3.2	5.9 ± 0.43	1.9 ± 0.02
C6ML3-17	- S - - Y Y R - - - -	4.9	3.4 ± 0.22	1.7 ± 0.02
100 mM HCl				
C6ML3-9	- S - - Y T - - - - -	1.0	7.6 ± 0.20	0.76 ± 0.03
C6ML3-14(2)	- - - - - P - W - - -	1.1	7.0 ± 0.40	0.77 ± 0.02
C6ML3-15	- - - - R P - W - - -	2.2	5.9 ± 0.80	1.3 ± 0.02
C6ML3-5(4)	- - - - Y - - - - - -	3.7	5.1 ± 0.34	1.9 ± 0.09

To determine if differences observed in the BIAcore were reflected in the affinity of selected scFv, a fourth round of selection was performed on the C6VLCDR3 phage antibody library. Phage were prepared from the third round of selection and elutions were performed using one of seven regimens: 1. 100 mM HCl; 2. 50 mM HCl; 3. 10 mM HCl; 4. 2.6 M MgCl$_2$; 5. 100 mM TEA; 6. 1 μM c-erbB-2 ECD; and 7. no elution (magnetic beads resuspended in 1.5 ml 1 M Tris HCl pH 7.4). After the fourth round of selection, only minor differences were observed in the frequency of ELISA positive scFv (*Table 3*). The titre of eluted phage, however, was 6 to 30 times lower when elutions were performed with MgCl$_2$, HCl, or TEA, compared to not eluting, or eluting with antigen (*Table 3*). To screen for the highest affinity scFv, native scFv was expressed from 24 ELISA positive clones and the dissociation rate constant (k$_{off}$) determined without purification. scFv was purified from the eight clones with the lowest k$_{off}$ from each of the seven elution regimens, the k$_d$, k$_{on}$, and k$_{off}$ determined, and the scFv gene sequenced (*Table 4*). scFv resulting in elutions with 50 mM HCl, 100 mM HCl, and 100 mM TEA had significantly lower K$_d$ than scFv resulting from elutions with 10 mM HCl, 1 μM c-erbB-2 ECD, or no elution (*Table 3*, *Table 4*). Elution with 100 mM HCl resulted in selection of the two highest affinity clones (*Table 4*, C6ML3-9 and C6ML3-14), however the difference in average affinity between elution with 100 mM HCl, 50 mM HCl, or 100 mM TEA was not statistically significant (*Table 3*). The different eluents, however, did yield scFv with similar kinetic properties but different sequences in V$_L$ CDR3 (*Table 4*).

Discussion

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

higher antigen concentration, or a conservative change in the antigen concentration used for the subsequent round.

As an alternative to monitoring selection using BIAcore, the titre of eluted phage is frequently followed, a rising titre being indicative of positive selection[10,18,23]. In this and previous work[14,26], however, we observed that positive selection on the basis of affinity occurred despite falling titres of eluted phage. When the titre of eluted phage did increase, there was a loss of binding phage, as determined by both BIAcore and ELISA. The mechanism for the increase in titre is unclear, however the majority of these phage have deleted at least one portion of the scFv gene. This could result in increased infection efficiency, due to the greater number of wild type pIII on the phage surface, or reduced toxicity to *E. coli* from leaky scFv expression. Regardless, following the titre of eluted phage is not a useful monitor of selection.

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

Our results also demonstrate the important effect of eluent choice on the affinities of selected antibodies, even when using limiting antigen concentration and BIAcore screening to identify the highest affinity scFv. Two previously described elution regimens were found to be the least effective for selecting higher affinity antibodies; infecting without elution by adding magnetic beads with antigen-bound phage directly to *E. coli* cultures[23], and competitive elution of scFv with soluble antigen[10,15,16]. When eluting by incubating phage bound to antigen with *E. coli*, the phage probably must dissociate from antigen for infection to occur. Steric hindrance, due to the size of paramagnetic beads, blocks the attachment of pIII on antigen bound phage to the f-pilus on *E. coli*. This would result in preferential selection of scFv with rapid k$_{off}$, consistent with our results. Since a reduction in k$_{off}$ is the major mechanism for decreases in K$_d$, this results in the selection of lower affinity scFv. Eluting with soluble antigen has a similar effect of the kinetics of selected scFv. The phage must first dissociate from immobilized antigen, then rebinding is blocked by binding of the phage to soluble antigen. Phage antibodies with the lowest k$_{off}$ will remain bound to immobilized antigen and therefore are not available for infection of *E. coli*.

The optimal type of eluent (acidic, basic, chaotropic) and concentration required will depend on the phage antibody affinity[27,28] and the type of bonds that need to be interrupted. This will vary considerably between libraries, depending on the nature of the antigen–antibody interaction. In this example, significantly higher affinity scFv were obtained eluting with HCl, pH 1.3 compared to HCl, pH

2.0. In fact, the affinities of scFv isolated after elution with HCl, pH 2.0 were no different than results obtained without eluting. Similarly, we studied 2.6 M $MgCl_2$, because we had previously determined it would remove 100% of bound wild type C6.5[24]. This concentration of $MgCl_2$, however, was ineffective in eluting C6.5 V_L CDR3 mutants. Eluting with higher concentrations of $MgCl_2$ would have resulted in the selection of higher affinity scFv. For example, 3 M $MgCl_2$ was required to elute 100% of C6L1 scFv ($K_d = 2.5 \times 10^{-9}$ M)[14] from a c-erbB-2 ECD BIAcore sensor chip and 4 M $MgCl_2$ was required to elute 100% of C6ML3-9 ($K_d = 1.0 \times 10^{-9}$ M).

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

An alternative to eluting with stringent solutions is to use antigen biotinylated with NHS-SS-Biotin (Pierce)[7]. All of the bound phage can be released from the magnetic beads by reducing the disulfide bond between antigen and biotin. One advantage of this approach is that elution of all phage is guaranteed. Use of NHS-SS-Biotin could be combined with use of a milder eluent for washing (determined by BIAcore analysis) to increase enrichment for higher affinity phage antibodies. Our results suggest, however, that use of stringent eluents that are chemically different (acidic, basic, or chaotropic) results in the selection of scFv of equally high affinity, but of different sequence. Isolation of scFv of different sequences has a number of advantages. Single amino acid changes can affect expression levels in *E. coli* dramatically[29]. For example, expression level of C6ML3-5 (100 μg l^{-1}) was 100 times less than for wild type C6.5 (10 mg l^{-1}). Furthermore, different scFv might have different physicochemical characteristics (dimerization, stability, or immunoreactivity) or even different effects *in vivo* (specificity, biodistribution, or clearance). Thus parallel selections using different stringent eluents should result in a greater number of high affinity binders than use of a single eluent.

The K_d of C6.5 was decreased 16 fold from a single library of V_L CDR3 mutants. This is at least twofold greater than the three to eightfold decreases in K_d previously obtained from a single library for protein binding phage antibodies. We conclude that this greater efficiency in affinity maturation results from use of the BIAcore to monitor and guide selections, and use of the optimal eluent,

rather than the specific CDR selected for mutagenesis[12,13]. For example, using BIAcore guidance, the K_d of C6ML3-9 was reduced an additional ninefold by randomizing four amino acids in V_H CDR3[38]. Use of the BIAcore to guide selections should decrease the number of libraries required to achieve the desired K_d.

Nomenclature

AMP	ampicillin
CDR	complementarity determining region
c-erbB-2 ECD	extracellular domain of c-erbB-2
cfu	colony forming units
ELISA	enzyme linked immunosorbent assay
GLU	glucose
HBS	hepes buffered saline, 10 mM hepes, 150 mM NaCl, pH 7.4
IPTG	isopropylβ–D–thiogalactopyranoside
k_{on}	association rate constant
k_{off}	dissociation rate constant
MPBS	2% skimmed milk powder in PBS
MTBS	2% skimmed milk powder in TPBS
PBS	phosphate buffered saline, 25 mM NaH_2PO_4, 125 mM NaCl, pH 7.0
RU	resonance units
scFv	single-chain Fv fragment
SPR	surface plasmon resonance
TEA	triethylamine
TPBS	0.05% v/v Tween 20 in PBS
V_H	immunoglobulin heavy chain variable region
V_L	immunoglobulin light chain variable region

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Appendix 2

Increased Affinity Leads to Improved Selective Tumor Delivery of Single-Chain Fv Antibodies[1]

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ABSTRACT

M_r 25,000 single-chain Fv (scFv) molecules are rapidly eliminated from the circulation of immunodeficient mice, yielding highly specific retention of small quantities of scFv in human tumor xenografts. We postulated that the specific retention of scFv in tumor could be enhanced by engineering significant increases in the affinity of the scFv for its target antigens. Affinity mutants of the human anti-HER2/*neu* (c-erbB-2) scFv C6.5 were generated by site-directed mutagenesis, which target the same antigenic epitope with a 320-fold range in affinity (3.2×10^{-7} to 1.0×10^{-9} M). *In vitro*, the K_d of each scFv correlated closely with the duration of its retention on the surface of human ovarian carcinoma SK-OV-3 cells overexpressing HER2/*neu*. In biodistribution studies performed in *scid* mice bearing established SK-OV-3 tumors, the degree and specificity of tumor localization increased significantly with increasing affinity. At 24 h after injection, tumor retention of the highest affinity scFv was 7-fold greater than that of a mutant with 320-fold lower affinity for HER2/*neu*. Because the rapid renal clearance of scFv may blunt the impact of improved affinity on tumor targeting, the distributions were also assayed in the absence of renal clearance (*e.g.*, in mice rendered surgically anephric). In this model, the peak tumor retentions of the two higher affinity scFv approximated that reported previously for IgG targeting the same SK-OV-3 tumors in *scid* mice with intact kidneys. In contrast, the mutant with the lowest affinity for HER2/*neu* failed to accumulate in tumor, indicating the presence of an affinity threshold that must be exceeded for active *in vivo* tumor uptake. These results indicate that affinity can significantly impact the *in vivo* tumor-specific retention of scFv molecules.

INTRODUCTION

The intrinsic properties of immunoglobulin molecules and their fragments regulate the *in vivo* biodistribution properties of these molecules in tumor-bearing hosts. Intact IgG molecules are large (M_r 150,000) glycoproteins that exhibit a slow systemic clearance, leading to poor tumor targeting specificity (1). Furthermore, IgG intratumoral diffusion is limited by size to a penetration rate of 1 mm every 2 days, potentially resulting in heterogeneous deposition in tumor (2). Smaller antibody-derived molecules include enzymatically produced M_r 50,000 Fab fragments and engineered M_r 25,000 scFv[3] consisting of

the heavy and light chain variable regions (V_H and V_L) connected by a flexible peptide linker (3, 4). Compared to IgG molecules, Fab and scFv exhibit significantly improved tumor specificity (5–7) and intratumoral penetration (8, 9) in animal models. However, the rapid blood clearance and monovalent nature of these small molecules result in significantly lower quantitative tumor retention of scFv and Fab fragments (5, 7), limiting their potential as carrier molecules of cytotoxic agents such as radioisotopes for radioimmunotherapy. Use of higher affinity scFv, with slower dissociation rate constants (k_{off}), could lead to an increased quantitative retention in tumor. However, no previous studies have compared the effects of affinity modulation on tumor targeting of immunoglobulin-based molecules that recognize identical epitopes of the same antigen.

Recently, it has proven possible to significantly increase antibody fragment affinity and decrease k_{off} using phage display (reviewed in Refs. 10 and 11). This has been accomplished by creating mutant scFv gene repertoires and displaying their encoded proteins on the surface of filamentous bacteriophage; higher affinity binders can then be selected on antigen. Using this approach, we have created affinity mutants of a human scFv (C6.5) that binds to an epitope on the extracellular domain of HER2/*neu* (12). The mutant scFv differ from each other by one to three amino acids, bind the same HER2/*neu* epitope, and span a 320-fold range of affinities (3.2×10^{-7} M to 1.0×10^{-9} M) and a 170-fold range of k_{off} ($1.3 \times 10^{-1} s^{-1}$ to $7.6 \times 10^{-4} s^{-1}$; Ref. 13). For the work described here, these C6.5-based scFv affinity mutants were used to examine the effect of affinity and k_{off} on *in vitro* and *in vivo* tumor retention of scFv.

MATERIALS AND METHODS

Production of C6.5 scFv and C6.5-based scFv Affinity Mutants. C6.5 is a HER2/*neu* binding human scFv isolated from a nonimmune phage antibody library (12, 14). C6G98A was identified during alanine scanning of the C6.5 V_H CDR3 and differs from C6.5 by one amino acid, having a glycine to alanine substitution at V_H residue 98 (13). C6ML3-9 was isolated from a phage antibody library of C6.5 mutants and differs from C6.5 by three amino acid substitutions in the V_L CDR3 (13). For all studies, native scFv was expressed (15) from *Escherichia coli* harboring the appropriate phagemid and purified from the bacterial periplasm (16) using immobilized metal affinity chromatography (17), followed by FPLC size-exclusion chromatography on a Superdex 75 HR 10/30 column (Pharmacia, Uppsala, Sweden), as described previously (13). The scFv resulting from the above procedures were found to be monomeric when evaluated by HPLC size exclusion chromatography on a Superdex 75 HR 10/30 column (Pharmacia). The K_d of C6.5, C6G98A, and C6ML3-9 were calculated from association (k_{on}) and dissociation (k_{off}) rate constants measured on immobilized HER2/*neu* ECD using surface plasmon resonance on a BIAcore instrument (Pharmacia) as described previously and reported (12, 13). To confirm the BIAcore measurements, the K_d for HER2/*neu* as expressed on SK-OV-3 cells was also measured by Scatchard analysis using radiolabeled C6.5 (12). In some assays, the scFv molecules were biotinylated using a commercial kit (Pierce, Rockford, IL). Prior to their use, the immunoreactivity of each biotinylated scFv was determined to be unchanged from the native form by measurement of binding kinetics by surface plasmon resonance as described above. 26-10 scFv, specific for digoxin, was generously provided by Dr. James Huston (Creative BioMolecules, Hopkinton, MA) for use as an irrelevant control.

Epitope Retention Assay. The ability of an unlabeled C6.5 affinity mutant to displace biotinylated C6.5 was determined using flow cytometry. One microgram of biotinylated C6.5 was added along with unlabeled scFv (C6.5, C6ML3–9, C6G98A, or 26-10) at a competitor:C6.5-biotin molar ratio of 1:1 or 10:1 to 1 × 10⁶ SK-OV-3 human ovarian carcinoma cells (#HTB 77; American Type Culture Collection, Rockville, MD), which overexpress HER2/neu. Reactions were performed in a total volume of 100 μl of FACS buffer (PBS with 0.1% sodium azide, pH 7.2). After a 15-min incubation at 37°C, the cells were washed two times with 2 ml of FACS buffer and fixed by the addition of 100 μl of 1% paraformaldehyde. Fifty μl of a 1:800 dilution of streptavidin-phycoerythrin (TAGO, Inc., Burlingame, CA) were then added to each tube; the samples were incubated for 15 min at 4°C and then washed two times with FACS buffer. The degree of fluorescence was determined by analysis on a FACScan flow cytometer (Becton Dickinson, San Jose, CA) as described (18).

Cell Surface Retention Assay. To assess the impact of affinity on the retention of the scFv affinity mutants with cell-bound HER2/neu, an in vitro cell surface retention assay was performed. In this assay, 0.1% sodium azide was included to prevent artificially prolonged retention of the scFv-biotin resulting from internalization. Twelve μg of biotinylated scFv was incubated with 1.2 × 10⁷ SK-OV-3 cells in a total volume of 0.5 ml of FACS buffer for 30 min at room temperature. The cells were centrifuged at 500 × g for 5 min at 4°C, washed with 10 ml of ice-cold FACS buffer two times, and then resuspended gently in 12 ml of FACS buffer at 37°C. The cell suspensions then were incubated at 37°C with gentle shaking in a water bath. To decrease the rebinding of dissociated biotinylated scFv to the cells, at 15, 30, 45, 60, 90, and 120 min after commencing the incubation, the suspensions were pelleted at 500 × g, the supernatants were aspirated, and the cells were gently resuspended in fresh FACS buffer (37°C). Immediately after each round of pelleting and resuspension, 0.5-ml aliquots containing 5 × 10⁵ cells were removed in triplicate (i.e., at 0, 15, 30, 45, 60, 90, and 120 min), placed on ice for 5 min and centrifuged at 500 × g for 5 min at 4°C. After removing the supernatants from the aliquots, the cells were gently resuspended in 50 μl of ice-cold FACS buffer containing 50 μl of a 1:800 dilution of streptavidin-PE, incubated on ice for 30 min, and washed twice with FACS buffer at 4°C. The cells were fixed with 1% paraformaldehyde, and the degree of fluorescence was determined by analysis on a FACScan flow cytometer as described above.

Radioiodination. All scFv were labeled with ¹²⁵I using the CT method as described previously (19). Briefly, 1.0–2.0 mg of protein were combined with ¹²⁵I (NEZ033H; DuPont NEN, Wilmington, DE) at an iodine:protein ratio of 1:10. Ten μg of CT (Sigma Chemical Co., St. Louis, MO) were added per 100 μg of protein, and the resulting mixture was incubated for 3 min at room temperature. Unincorporated radioiodine was separated from the labeled protein by gel filtration using the G-50–80 centrifuged-column method (20). The final specific activity of the ¹²⁵I-scFv molecules were about 1.0 mCi/mg. The quality of the radiopharmaceuticals was evaluated by high-performance liquid chromatography, SDS-PAGE, and in a live cell-binding assay. The high-performance liquid chromatography analysis was performed using a Superdex 75 column (Pharmacia). Eluted fractions were collected and counted in a gamma well counter (Beckman) (5). The elution profiles consistently demonstrated that greater than 99% of the radioactivity was associated with the protein peak. The radioiodinated scFv molecules were evaluated by SDS-PAGE. Reduced and nonreduced SDS-PAGE were run using 12% gels (10 × 12 cm; 12D0.75HMC-10P; Jule, Inc., New Haven, CT; Refs. 5 and 21). Migration of the scFv was detected by autoradiography at −70°C using Kodak X-ray film with Kodak X-Omatic regular intensifying screens. Greater than 98% of the nonreduced ¹²⁵I-scFv preparations migrated on SDS-PAGE as approximately M_r 26,000, indicating their monomeric nature. The immunoreactivity of the radiopharmaceuticals was determined in a live cell-binding assay using HER2/neu expressing SK-OV-3 cells (5). Ten ng of labeled scFv in 100 μl of PBS were added in triplicate to 5 × 10⁶ SK-OV-3 cells in 15 ml of polypropylene centrifuge tubes. The cells were incubated for 30 min at room temperature, washed with 2.0 ml of PBS, and centrifuged for 5 min at 500 × g. Supernatants were separated from the cell pellets; both were transferred to 12 × 75 counting tubes, and the percentage of activity associated with the cell pellet was determined. The results of the live cell-binding assays indicated that the radioiodinated scFv were still reactive with cell surface HER2/neu. The activity associated with the cell pellet was 61.2, 59.0, 5.3, and 2.7%, respectively, for C6ML3–9, C6.5, C6G98A, and 26-10. The poor retention of the

C6G98A was consistent with its rapid dissociation rate from the HER2/neu antigen.

Biodistribution Studies. Four to 6-week-old inbred C.B17/Icr-scid mice or outbred Icr:Ha(ICR)-scid mice were obtained from the Fox Chase Cancer Center Laboratory Animal Facility. SK-OV-3 cells (2.5 × 10⁶) in log phase were implanted s.c. on the abdomens of the mice. After 6–8 weeks, the tumors had achieved sizes of ~100 mg, and Lugol's solution was placed in the drinking water to block thyroid accumulation of radioiodine. Two days later, biodistribution studies were initiated.

Initial studies were performed with escalating doses to determine the quantity of each scFv required to saturate the tumor antigen pool. Doses of 5, 25, 125, or 625 μg of radioiodinated C6.5, C6ML3–9, or 26–10 scFv (negative control) were selected based upon our previous observation that a 125-μg dose of the C6.5 scFv is capable of saturating the tumor antigen pool.[4] The scFv molecules were administered by tail vein injection to cohorts of three tumor-bearing outbred scid mice. The mice were sacrificed at 4 h after injection, and the tumors and organs were removed, weighed, and counted in a gamma counter to determine the percentage of the injected dose localized per gram of tissue (%ID/g) for each labeled scFv (5, 22). The degree of tumor-specific retention was determined by subtracting the value of the 26-10 (irrelevant) tumor:blood ratios from that observed with each dose group for the anti-HER2/neu scFv molecules. Saturation was defined as the dose above which specific retention did not increase.

To study the impact of affinity on tumor retention, ¹²⁵I-scFv (C6.5, C6ML3–9, C6G98A, or 26-10) was administered to cohorts of six inbred scid mice by tail vein injection at the dosage determined above to just saturate the tumor antigen with C6ML3–9 (25 μg). 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 determined immediately after injection and just prior to euthanization. Groups of six mice were sacrificed at 4 and 24 h after injection; tumor, organ, and blood retentions were determined as described above. The mean and SE for each group of data were calculated, and T:O ratios were determined. Significance levels were determined using the Wilcoxon rank sum test.

Additional studies were performed on surgically nephrectomized mice to determine the impact of affinity on biodistribution in the absence of renal clearance. Male scid mice bearing established s.c. SK-OV-3 tumors were lightly anesthetized with metofane (Mallinckrodt Veterinary, Mundelein, IL), and contralateral retroperitoneal incisions were made. The kidneys were identified, ligated with 4-0 silk, and excised from the mice with special care taken to prevent damage to the adrenal blood supply. The incisions were then closed with surgical staples. Biodistribution studies were initiated 1 h later. Twenty μg of ¹²⁵I-scFv (C6.5, C6ML3-9, or C6G98A) were administered to cohorts of three nephrectomized, inbred scid mice by tail vein injection. Total injected doses were determined as described above. Blood samples and whole-body counts of the mice were collected immediately after injection and just prior to euthanization. Mice were sacrificed at 4 and 24 h after injection, and tumor, organ, and blood retentions were determined as described above. The mean and SE for each group of data were calculated, and T:O ratios were determined. Significance levels were determined using the Wilcoxon rank sum test.

RESULTS

Amino Acid Sequences and Affinity Constants of C6.5-derived scFv Molecules. The amino acid sequences of C6G98A and C6ML3-9 differ from each other and from the C6.5 scFv from which they were derived by only a few amino acids in the V_H and V_L CDR3 domains (Table 1). The binding kinetics (k_{on} and k_{off}) of C6G98A, C6.5, and C6ML3-9 for HER2/neu ECD were determined using surface plasmon resonance, and the results were used to calculate the K_d (Table 1). The K_d of the parental molecule (C6.5) was 1.6 × 10⁻⁸ M, which was in close agreement to the K_d of 2.0 × 10⁻⁸ M determined previously by Scatchard analysis for radiolabeled C6.5 binding to HER2/neu expressing SK-OV-3 cells (12). Mutant K_d ranged from

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[4] Unpublished data.

Table 1 *Affinity and binding kinetics of C6.5-based scFv*

k_{on} and k_{off} were measured for purified scFv by surface plasmon resonance, and the K_d was calculated as k_{off}/k_{on}. The three C6.5-derived scFv molecules differ only in the sequences of V_H or V_L CDR3.

scFv clone	K_d (M)	k_{on} (M^{-1}s^{-1})	k_{off} (s^{-1})	Amino acid sequence	
				V_H CDR3	V_L CDR3
C6.5	1.6×10^{-8}	4.0×10^{-5}	6.3×10^{-3}	HDVGYCSSSNCAKWPEYFQH	AAWDDSLSGWV
C6ML3-9	1.0×10^{-9}	7.6×10^{-5}	7.6×10^{-4}	-------------------------------	-S–YT---------------
C6G98A	3.2×10^{-7}	4.1×10^{5}	1.4×10^{-1}	---A-------------------------------	-------------------------

20-fold less than C6.5 (C6G98A, $K_d = 3.2 \times 10^{-7}$ M) to 16-fold greater than C6.5 (C6ML3-9, $K_d = 1.0 \times 10^{-9}$ M), resulting in a 320-fold range of affinities and a 170-fold range of k_{off}.

Determination of Epitope Conservation. To ensure that the fine specificity of C6.5 mutants was maintained, a competition assay was performed. In this assay, biotinylated C6.5 was mixed with each unlabeled mutant scFv in either a 1:1 or 1:10 molar ratio. Following an incubation with SK-OV-3 cells overexpressing HER2/*neu*, the amount of C6.5-biotin bound to the surface of the cells was determined by flow cytometry. Each C6.5 mutant successfully competed with C6.5-biotin for the target antigen, whereas the 26-10 scFv negative control had no effect (Table 2). As expected, the highest affinity C6.5 mutant (C6ML3-9) displayed significant binding competition at even the lowest molar ratio. C6G98A, the scFv with the lowest affinity, required the highest molar ratios to compete with biotinylated C6.5.

In Vitro Cell Surface Retention. The *in vitro* cell surface retentions of biotinylated C6.5 and C6ML3-9 were determined, both to verify the observed differences in k_{off} and to confirm that binding to the antigen in the BIAcore instrument had biological significance. The lowest affinity clone, C6G98A, was not assayed *in vitro* because it exhibited a dissociation from the cell surface that was too rapid to quantitate by flow cytometry. SK-OV-3 cells were saturated with biotinylated scFv and washed to remove unbound scFv, and the cells were diluted in buffer at 37°C. The cells were washed at intervals to remove dissociated scFv-biotin, and aliquots were removed, fixed with paraformaldehyde to prevent dissociation of the bound scFv-biotin, and incubated with streptavidin-PE. Samples were analyzed by flow cytometry to determine mean fluorescence intensity values and the associated percentages of biotinylated scFv bound to the cell surfaces. Representative results from one of two separate experiments are presented in Fig. 1. The half-life ($t_{1/2}$) of the scFv on the cell surface was approximately 2.5 min for C6.5 and 11 min for C6ML3-9. The 26-10 control scFv did not bind to the cells. These values agree closely with the $t_{1/2}$ calculated from the k_{off} as determined by surface plasmon resonance (1.6 min for C6.5 and 13 min for C6ML3-9), assuming a simple exponential decay (e^{-kt}, where $k = k_{off}$).

Biodistribution Studies. Preliminary biodistribution studies were performed to determine the dose of scFv required for tumor antigen
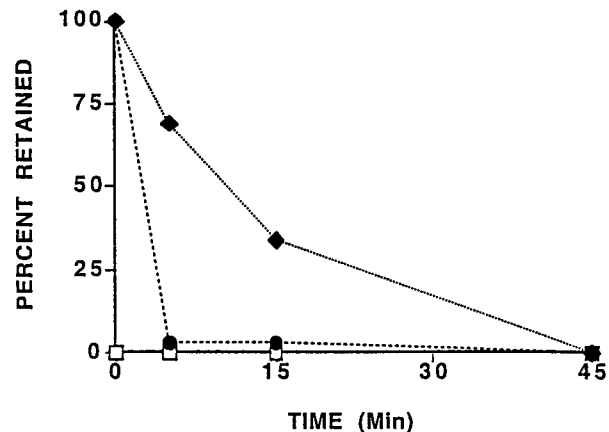

Fig. 1. *In vitro* cell surface retention assay. The cell surface retention of biotinylated C6.5 (●), C6ML3-9 (◆), and 26-10 scFv (□) was determined by saturating SK-OV-3 cells with scFv and incubating the cells at 37°C in a shaker bath. At the indicated times, the cells were pelleted and resuspended in FACS buffer, and aliquots were removed. The aliquots were fixed, treated with streptavidin-PE, and the mean fluorescent intensity was determined by flow cytometry. Values are expressed as a percentage of initial mean fluorescent intensity.

saturation. Escalating doses of radioiodinated anti-HER2/*neu* C6.5, C6ML3-9, or the irrelevant 26-10 scFv were administered to tumor-bearing *scid* mice. The mice were sacrificed at 4 h, after about one elimination phase half-life ($t_{1/2}\beta$), and tumor:blood ratios were determined. The dose of each anti-HER2/*neu* scFv required to saturate the tumor antigen pool was determined after subtracting the activity attributable to blood and extravascular spaces as defined by the irrelevant 26-10 scFv. Saturation of the HER2/*neu* on the SK-OV-3 tumors within the first 4 h after injection occurred at doses of 25 μg (C6ML3-9) and 125 μg (C6.5).

Twenty-four-h biodistribution studies then were performed in *scid* mice bearing s.c. SK-OV-3 tumors to examine the role of affinity on the specificity and degree of tumor retention. These assays used ^{125}I-labeled forms of C6G98A, C6.5, C6ML3-9, and a negative control scFv (26-10) at the dose of 25 μg, which was determined above to achieve early tumor saturation with C6ML3-9. The HER2/*neu*-specific scFv were selected to provide the following stepwise increase in affinity; C6G98A (3.2×10^{-7} M), C6.5 (1.6×10^{-8} M), and C6ML3-9 (1.0×10^{-9} M). The biodistribution studies revealed a correlation between the affinity and the %ID/g of the radioiodinated scFv retained in tumor (Table 3). The greatest degree of tumor retention was observed with ^{125}I-C6ML3-9 (1.42 ± 0.23 %ID/g). At 24 h, significantly less tumor retention was achieved with ^{125}I-C6.5 (0.80 ± 0.07 %ID/g) and C6G98A (0.19 ± 0.04 %ID/g). The tumor retention of the lowest affinity clone ^{125}I-C6G98A was significantly less than that of C6.5 ($P = 0.00001$) and was identical to that of the negative control ^{125}I-26-10. The T:O ratios also reflected the greater retention of higher-affinity species in tumor (Table 3). For example, tumor:blood ratios of 17.2, 13.3, 3.5, and 2.6 and tumor:liver ratios of 26.2, 19.8, 4.0, and 3.1 were observed for C6ML3-9, C6.5, C6G98A, and 26-10, respectively, at 24 h.

We postulated that the rapid systemic elimination of the small scFv

Table 2 *Epitope retention assay*

The retention of epitope specificity of C6.5-based scFv affinity mutants was determined by testing their abilities to compete with biotinylated C6.5 for HER2/*neu* expressed on SK-OV-3 cells. The amount of C6.5-biotin retained was determined by flow cytometry after the addition of streptavidin-PE. Results for each competitor are expressed as a percentage of the binding observed in the absence of competitor. 26-10 is an irrelevant control scFv that binds digoxin.

Competitor scFv	% of maximal binding by C6.5-biotin	
	1:1[a]	10:1[a]
C6G98A	100%	64.0%
C6.5	42.7%	5.9%
C6ML3-9	6.9%	0.5%
26-10	100%	100%

[a] Molar ratio.

Table 3 *Twenty-four-h biodistribution of ^{125}I-labeled C6.5 and its affinity mutants in tumor-bearing scid mice*

Twenty-five μg of iodinated scFv were administered to SK-OV-3 tumor-bearing *scid* mice by i.v. tail vein injection. Cohorts of six mice were sacrificed at 24 h after injection, and the %ID retained per gram of tissue or mL of blood was determined and the T:O ratios calculated. Values are presented for major organs of interest. Unless otherwise indicated, SEs were ≤36% of the indicated value.

| scFv | 26-10 | | C6G98A | | C6.5 | | C6ML3-9 | |
| | NA[a] | | 3.2×10^{-7} | | 1.6×10^{-8} | | 1.0×10^{-9} | |
K_d Organs	%ID/g	T:O	%ID/g	T:O	%ID/g	T:O	%ID/g	T:O
Organs								
Tumor	0.18		0.19		0.80[b]		1.42[c,d]	
Blood	0.07	2.6	0.06	3.53	0.06	13.3	0.09	17.2
Liver	0.06	3.1	0.10[e]	3.95	0.05	19.8	0.07	26.2
Spleen	0.09	2.0	0.06	4.0	0.06	16.4	0.08	23.9
Lung	0.08	2.2	0.05	4.17	0.06	13.1	0.09	17.6
Kidney	0.16	1.1	0.22	0.85	0.11	7.5	0.152	9.7
Stomach	0.18	1.1	0.18	1.8	0.15	7.8	0.35	6.3
Muscle	0.02[d]	9.3	0.02[d]	11.1	0.05[d]	40.6	0.03[d]	59.1

[a] NA, not applicable.
[b] $P < 0.05$ compared to C6G98A and 26-10.
[c] $P = 0.001$ compared to C6G98A and 26-10.
[d] $P = 0.025$ compared to C6.5.
[e] SE ≤ 50% of the value.

molecules would limit their perfusion of the tumors, thus hindering the assessment of the role of affinity on tumor targeting. Accordingly, biodistributions were performed with ^{125}I-labeled C6.5, C6G98A, and C6ML3-9 in surgically nephrectomized mice to determine the impact of affinity in the absence of renal elimination. Although blood and normal organ retentions were similar for all three scFv molecules, the rate of accumulation in tumor was closely correlated with affinity for HER2/*neu* (Table 4). Tumor retention of C6ML3-9 scFv peaked at 17.3 %ID/g by 4 h after administration and remained constant through the end of the study, whereas C6.5 scFv accumulated in the tumors during the course of the study, peaking between the 4- and 24-h time points. The lowest affinity scFv, C6G98A, failed to accumulate in tumor over time and remained at below 10 %ID/g throughout the assay. However, in a separate biodistribution study performed in nephrectomized *scid* mice, the 24-h tumor retention of C6G98A was found to be 10-fold greater than that observed with the 26-10 negative control scFv (data not shown). These results suggest that the continued presence of C6G98A is capable of overcoming its low affinity, leading to the active maintenance of measurable tumor concentrations.

DISCUSSION

This is the first reported examination of the effects of affinity on tumor targeting by antibody based molecules that bind to the same epitope and antigen. The C6.5 scFv and its affinity mutants differed from each other by only one to three amino acid residues, yet differed in affinity for the same epitope of HER2/*neu* by 320-fold. The differences in K_d among the affinity variants are due almost entirely

to differences in k_{off}. As expected, the cell surface retention properties of these molecules (Fig. 1) demonstrate the relevance of k_{off} measurements by surface plasmon resonance to binding characteristics on the cell surface. Although the lowest affinity mutant (C6G98A, $K_d = 3.2 \times 10^{-7}$ M) was not used in the cell surface retention assay because it exhibited an off-rate that was too rapid to measure by flow cytometry, its ability to bind to cell surface HER2/*neu* was demonstrated by competition with biotinylated C6.5 in the epitope conservation assay (Table 2). In mice, the 24-h tumor retention correlated directly with the affinities of the molecules. Targeting of the lowest affinity C6.5 mutant (C6G98A, $K_d = 3.2 \times 10^{-7}$ M) was no different than that of an irrelevant control scFv, whereas C6ML3-9 ($K_d = 1.0 \times 10^{-9}$ M) had a higher %ID/g tumor at 24 h than C6.5 ($K_d = 1.6 \times 10^{-8}$ M) or C6G98A. Tumor:normal organ ratios also increased with increasing affinity. These data indicate that, over the range of affinities studied in this model, increasing affinity improves the selective targeting of scFv to solid tumors.

The potential impact of affinity on the successful targeting and retention of MoAb in tumors has led to two disparate theories. Fujimori *et al.* (23) has postulated that high affinity MoAb will not successfully penetrate deeply into tumors due to a binding site barrier effect, in which interaction with the first antigen encountered at the periphery of the tumor will block further diffusion of the MoAb into the tumor. Support for this hypothesis comes from the observation that i.v. administration of the MoAb ^{131}I-D3, specific for the chemically induced L10 carcinoma line ($K_a = 1.6 \times 10^{10}$ M^{-1}) to guinea pigs bearing intradermal L10 tumors, resulted in a peripheral binding to

Table 4 *Biodistribution of C6.5-based scFv molecules in anephric tumor-bearing scid mice*

Twenty μg of ^{125}I-labeled C6G98A ($K_d = 3.2 \times 10^{-7}$ M), C6.5 ($K_d = 1.6 \times 10^{-8}$ M) or C6ML3-9 ($K_d = 1.0 \times 10^{-9}$ M) were administered by i.v. tail vein injection to anephric *scid* mice bearing s.c. SK-OV-3 tumors ($n = 3$ mice/time point). Tissue and blood retention of the radiolabeled scFv molecules were determined as described in the text. Results are expressed as the %ID/g tissue (or mL blood). T:O ratios are presented in parentheses. SEs were less than 25% of the associated value, unless noted.

| | C6G98A | | C6.5 | | C6ML3-9 | |
| | 3.2×10^{-7} | | 1.6×10^{-8} | | 1.0×10^{-9} | |
K_d Organ	4 h	24 h	4 h	24 h	4 h	24 h
Tumor	9.3	8.3	12.7	21.1	17.3	17.3
Blood	11.8 (0.8)	7.0 (1.2)	12.4 (1.0)	7.1 (3.1)	9.9 (1.8)	5.0 (3.5)
Liver	4.5 (2.1)	4.3 (1.9)	3.5 (3.7)	3.7 (5.7)	3.6 (4.8)	3.3 (5.2)
Spleen	4.6 (2.0)	5.1 (1.6)	4.8 (2.7)	4.5 (5.0)	4.4 (4.0)	5.4 (3.2)
Lung	10.2 (0.9)	5.7 (1.4)	10.3 (1.2)	6.0 (3.6)	8.0 (2.3)	3.9 (4.5)
Stomach	10.8 (0.9)	21.7 (0.5)	7.8 (1.7)	20.7 (1.1)	8.1 (2.2)	29.2 (0.6)
Muscle	2.9 (3.2)	2.9 (2.8)	2.3 (5.7)	1.8 (12.2)	2.1 (8.4)	1.5 (16.1)[a]

[a] SE = 53% of the associated T:O ratio value.

antigenic patches, whereas ^{125}I-labeled control MoAb was uniformly distributed throughout the tumor (24). In contrast with the theory of Fujimori *et al.* (23), evidence for increased affinity prolonging the tumor retention of radiolabeled antibody comes from studies using B72.3, a MoAb with a specificity for the pancarcinoma antigen TAG-72. The tumor targeting of B72.3 was compared to a series of higher affinity second generation MoAbs, each reactive with a different epitope of TAG-72 (25). In this study, the second generation MoAb CC49 ($K_d = 6 \times 10^{-11}$) localized human tumor xenografts in athymic nude mice better than did the lower affinity molecule B72.3 ($K_d = 2 \times 10^{-9}$). However, in subsequent clinical trials, the second generation MoAb CC49 failed to display improved tumor retention over that observed for B72.3 (26). As the authors noted, however, it was unlikely that affinity was the only variable affecting the tumor localization properties. Other factors, such as accessibility and quantity of the targeted epitopes, may have influenced the results. Furthermore, different distribution patterns would likely be achieved with smaller species such as the scFv molecules used in the present study. Rigorous examination of this issue will require the study of antibody-based molecules of various sizes with a range of affinities for an identical epitope.

Despite the large (320-fold) increase in affinity, the actual amounts retained at 24 h for the highest affinity scFv (1.4% ID/g tumor) remain much less than those observed for IgG molecules in similar model systems. The improved quantitative targeting of IgG molecules in these animals has been thought to be related to the prolonged clearance of IgG in mice: typically, the $t_{1/2}\beta$ of IgG in mice is on the order of 107 h (27), whereas the $t_{1/2}\beta$ of most scFv is approximately 3.5 h (28). The rapid renal clearance of scFv may blunt the impact of improved affinity on tumor targeting by decreasing opportunities for these molecules to diffuse to tumor sites. Accordingly, we sought to examine the impact of affinity in a model system where clearance had been negated. This was accomplished by determining the biodistribution of labeled scFv in mice following the surgical excision of their kidneys.

The studies in anephric mice resulted in two observations of significance:

(*a*) The peak tumor retentions for the two higher affinity scFv approximated those observed with IgG targeting the HER2/*neu* antigen on the same SK-OV-3 tumors in *scid* mice with functional kidneys (21% ID/g for scFv, 20% for IgG; Ref. 29). The specificity of tumor retention was surprisingly good, despite the absence of renal clearance, and improved over time. Thus, if renal clearance can be negated, scFv molecules can target tumors with as much quantitative deposition as IgG molecules, and with greater specificity. Because their large size decreases their diffusion coefficients, IgG molecules are ill-suited to overcome the many morphological and physiological features that limit the accessibility of antibodies to tumors. For example, IgG will diffuse 100 μm in 1 h, 1 mm in about 2 days, and 1 cm in 7 months (2). Decreasing the size of antibody-based molecules increases their interstitial diffusion rates and tumor distribution. Because scFv are smaller and diffuse through tumors more efficiently, the targeting of tumor cells in malignant masses is likely to be more homogeneous than with IgG (8). These results are in agreement with Buchegger's observation of the impact of size on the maintenance of sufficient antibody fragment in circulation to achieve significant tumor targeting (30). Working with a similar nephrectomized mouse model, Buchegger *et al.* (30) reported significantly greater tumor retention of a radiolabeled Fab fragment as compared with the parent IgG.

(*b*) The second observation of significance from the studies in anephric mice is that higher affinity scFv do exhibit improved quantitative and selective tumor retention. The importance of affinity is

underscored by the minimal tumor retention achieved with the lowest affinity variant, C6G98A. Although the C6G98A did exhibit 10-fold greater tumor retention than the negative control 26-10 scFv, its low affinity was insufficient to mediate an accumulation in tumor over time. Thus, C6G98A never approached the peak tumor retentions achieved with C6ML3-9 and C6.5, clearly, an affinity threshold that must be crossed to achieve selective retention in the *in vivo* setting. In this model system, the *in vivo* threshold exceeds the *in vitro* threshold for reasons that are not clearly understood. When a molecule with 320-fold higher affinity was examined in the same model, its tumor retention was significantly higher, and tumor:blood ratios improved to 3.5 to 1, despite the absence of renal clearance of the molecule. Because these studies were performed using molecules radioiodinated by the CT method, substantial dehalogenation occurred, as evidenced by the large accumulation of radioiodine in the stomach at 24 h. Accordingly, estimates of tumor retention may be artificially low, not reflecting the presence of scFv that have lost their label (data not shown). This issue is being addressed by labeling modified scFv molecules using methodologies to create covalent associations between the radioiodine and the proteins (31); we have found that such strategies greatly improve the quantity and specificity of tumor targeting. The studies reported here used protein doses of scFv molecules that saturated available tumor antigen so that continued accumulation of the highest affinity scFv, C6ML3-9, may have been limited by this factor. However, peak tumor retention was achieved more rapidly, *e.g.*, after only 4 h, than with the lower affinity scFv, C6.5. Furthermore, this level of tumor retention persisted as the blood levels of C6ML3-9 dropped, yielding improved tumor:normal organ ratios at 24 h as compared with C6.5.

Advances in protein engineering techniques and the use of phage display make it possible to create small, human antibody-based targeting molecules, such as scFv, and engineer affinity to values not achievable by rodent immunization (11). For example, we have further increased the affinity of C6.5-based scFv another 100-fold to a $K_d = 1.3 \times 10^{-11}$ M (13). The *in vivo* tumor targeting properties of this and related molecules require study. The use of extremely high-affinity scFv molecules may further improve *in vivo* targeting, particularly when the k_{off} rates of the molecules exceed the $T_{1/2}\beta$ of the molecules in circulation. Given the results presented here, however, it is likely that rapid clearance will still impose a ceiling on the amount of administered protein that can accumulate at tumor sites. Strategies to retain small size and reduce renal clearance require examination. Alternatively, the scFv molecules can be used as building blocks to create small, bivalent, scFv-based molecules, such as diabodies. These molecules can bind bivalently to cell surface antigens, resulting in a large increase in functional affinity, but are still below the renal threshold and thus rapidly cleared from the circulation. For example, constructing a diabody from the V-genes of C6.5 improves affinity 40-fold ($K_d = 4.0 \times 10^{-10}$ M) and yields a 6-fold increase in specific tumor retention at 24 h compared to the C6.5 scFv (6.5% ID/g *versus* 1.0% ID/g for C6.5 scFv).[5] Improved quantitative delivery may also partially result from a longer $t_{1/2}\beta$ for the diabody (6.4 h *versus* 3.5 h for the scFv molecules). Construction of diabodies from the V-genes of higher affinity C6.5-based scFv molecules may lead to further improvements in specific targeting. These and other potential modifications to the structures of scFv will enhance the ability of these molecules to function as delivery vehicles that can achieve the needed balance of quantitative and selective tumor retention to be effective in cancer therapy strategies.

[5] G. P. Adams, R. Schier, A. M. McCall, R. S. Crawford, E. J. Wolf, L. M. Weiner, and J. D. Marks. Prolonged *in vivo* tumor retention of a human diabody targeting the extracellular domain of human HER2/*neu*, Br. J. Cancer, in press.

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Appendix 3

Prolonged in vivo tumour retention of a human diabody targeting the extracellular domain of human HER2/*neu*

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Summary Single-chain Fv (scFv) molecules exhibit highly specific tumour-targeting properties in tumour-bearing mice. However, because of their smaller size and monovalent binding, the quantities of radiolabelled scFv retained in tumours limit their therapeutic applications. Diabodies are dimeric antibody-based molecules composed of two non-covalently associated scFv that bind to antigen in a divalent manner. In vitro, diabodies produced from the anti-HER2/*neu* (c-erbB-2) scFv C6.5 displayed approximately 40-fold greater affinity for HER2/*neu* by surface plasmon resonance biosensor measurements and significantly prolonged association with antigen on the surface of SK-OV-3 cells ($t_{1/2}$ cell surface retention of > 5 h vs 5 min) compared with C6.5 scFv. In SK-OV-3 tumour-bearing *scid* mice, radioiodinated C6.5 diabody displayed a highly favourable balance of quantitative tumour retention and specificity. By as early as 4 h after i.v. administration, significantly more diabody was retained in tumour (10 %ID g^{-1}) than in blood (6.7 %ID ml^{-1}) or normal tissue (liver, 2.8 %ID g^{-1}; lung, 7.1 %ID g^{-1}; kidney, 5.2 %ID g^{-1}). Over the next 20 h, the quantity present in blood and most tissues dropped approximately tenfold, while the tumour retained 6.5 %ID g^{-1} or about two-thirds of its 4-h value. In contrast, the 24-h tumour retention of radioiodinated C6.5 scFv monomer was only 1 %ID g^{-1}. When diabody retentions were examined over the course of a 72-h study and cumulative area under the curve (AUC) values were determined, the resulting tumor–organ AUC ratios were found to be superior to those previously reported for other monovalent or divalent scFv molecules. In conclusion, the diabody format provides the C6.5 molecule with a distinct in vitro and in vivo targeting advantage and has promise as a delivery vehicle for therapeutic agents.

Keywords: diabody; single-chain Fv; tumour targeting; avidity; immunodeficient mice

A major goal of antibody-based cancer therapy has been to specifically deliver toxic payloads, such as radioisotopes, toxins or drugs, to tumours. The range of antibody binding site-based molecules includes IgM (1000 kDa), IgG (150 kDa), F(ab')$_2$ (100 kDa), Fab (50 kDa), (scFv')$_2$ (55 kDa) and scFv (25 kDa). In immunodeficient mice, larger molecules such as IgG and F(ab')$_2$ fragments are retained at high levels in human tumour xenografts with a low degree of specificity (Milenic et al, 1991; Adams et al, 1992), while smaller molecules such as scFv, (scFv')$_2$ and Fab are retained in tumour at comparatively lower levels with greatly improved specificity (Beaumier et al, 1985; Colcher et al, 1990; Milenic et al, 1991; Adams et al, 1993). The most prominent determinant of the above targeting properties is the size of the antibody-based molecule relative to the renal threshold for first-pass clearance. Another important feature of antibody-based molecules is valence, as significantly greater tumour retention has been associated with multivalent binding to target antigen (Milenic et al, 1991; Adams et al, 1993, 1996; Wolf et al, 1993). Recently, attention has focused upon the generation of divalent scFv-based molecules with molecular weights in the range of the renal threshold for first-pass clearance. These include 50-kDa diabodies (Holliger, 1993), 55-kDa (scFv')$_2$ (Adams et al, 1993), 60 to 65-kDa amphipathic helix-based scFv dimers (Pack et al, 1992, 1993) and 80-kDa (scFv-C$_H$3)$_2$ LD minibodies and Flex minibodies (Hu Shi-zhen et al, 1996). While each

of these proteins is capable of binding two antigen molecules, they differ in the orientation, flexibility and span of their binding sites.

In this report, we examine the potential of diabody molecules to function as vehicles for the specific, quantitative delivery of radioisotopes to tumours. Diabodies are scFv dimers in which each chain consists of a variable heavy (V$_H$) domain connected to a variable light (V$_L$) domain using a peptide linker that is too short to permit pairing between domains on the same chain (Holliger et al, 1993). Consequently, pairing occurs between complementary domains of two different chains, creating a stable non-covalently bound dimer with two binding sites (Figure 1) (Perisic et al, 1994). We have used the human anti HER2/*neu* (c-erbB-2) scFv C6.5 (Schier et al, 1995) to construct a C6.5 diabody. While HER2/neu expression on normal human tissues is limited, it is overexpressed in a number of cancers, including breast and ovarian carcinoma (King et al, 1985; Kraus et al, 1987; van de Vijver et al, 1987; Berchuck et al, 1990; Schier et al, 1995), gastric tumours and colon adenocarcinomas (Yokota et al, 1988). Its relevance as a target for antibody-based therapy is further underscored by the correlation of HER2/*neu* overexpression with a poor prognosis in several malignancies (Slamon et al, 1987; Allred et al, 1992). Here, we present the C6.5 diabody's in vitro binding characteristics and in vivo distribution in tumour-bearing *scid* mice.
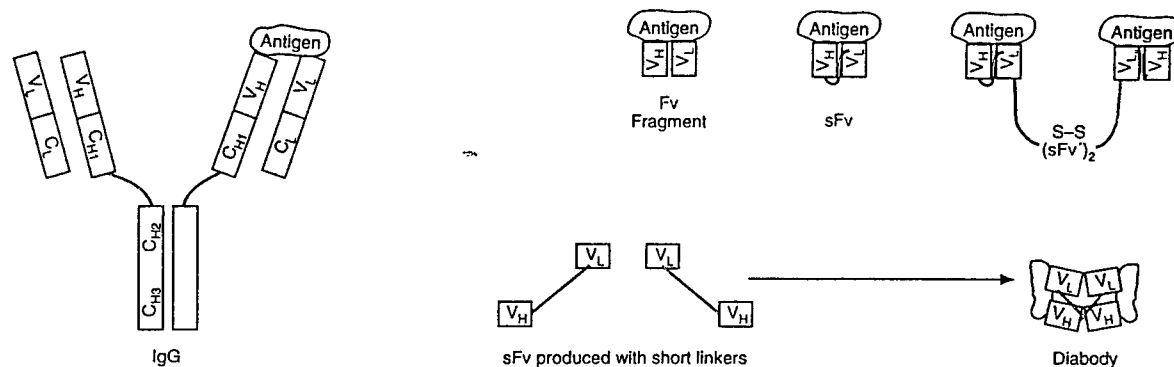
METHOD

C6.5 scFv and diabody production

The C6.5 scFv in pUC119mycHis was expressed from *E. coli* TG1 and purified by immobilized metal chelate chromatography

Figure 1   The structures of an IgG molecule (~ 150 kDa), an Fv fragment (~22 kDa), a recombinant scFv (~25 kDa) molecule, a dimeric (scFv')$_2$ molecule (~55 kDa) and a diabody molecule (~50 kDa) are presented. While they are identical, the two diabody components are illustrated with different shading to clarify the structure

(IMAC) followed by fast protein liquid chromatography (FPLC) size-exclusion chromatography using a Superdex 75 column as previously described (Schier et al, 1995). To create the C6.5 diabody, the C6.5 V_H and V_λ genes were joined together by polymerase chain reaction (PCR) splicing by overlap extension using an oligonucleotide that encoded a five amino acid linker (G_4S) between the C-terminus of the V_H and the N-terminus of the V_λ gene. First, the C6.5 V_H and V_λ genes were amplified using PCR from C6.5 scFv DNA using the primers LMB3 and DIAFOR (5'-CCA CCT GAG GAG ACG GTG ACC-3') (Marks et al, 1991) for the V_H gene and LMB2 and DIABACK (5'-GGT CAC CGT CTC CTC AGG TGG AGG CGG TTC ACA GTC TGT GTT GAC GCA GCC G-3') (Marks et al, 1991) for the V_λ gene. The V_H and V_λ genes were gel purified and 200 ng of each combined in a 50-μl reaction with 5 U of Vent DNA polymerase (New England Biolabs). The reaction mixture was cycled seven times to join the fragments (94°C for 1 min, 50°C for 1 min, 72°C for 1.5 min) after which 20 pM of the primers LMB2 and LMB3 were added, and the reaction cycled 25 times to amplify the products. The resulting diabody gene product was digested with NcoI and NotI, gel purified and ligated into NcoI/NotI-digested pUC119mycHis (Schier et al, 1995). The ligation mixture was used to transform E. coli TG1, and clones containing the correct insert identified by PCR screening and DNA sequencing. Native diabody was expressed (Breitling, 1991) and purified from the bacterial periplasm using IMAC (Hochuli et al, 1988) followed by FPLC size-exclusion chromatography using a Superdex 75 column.

Measurement of C6.5 scFv and diabody affinity for c-erbB-2

The affinities of C6.5 scFv and C6.5 diabody for the HER2/neu ECD were determined using surface plasmon resonance in a BIAcore (Pharmacia, Sweden) generally as previously described (Schier et al, 1995, 1996). In a BIAcore flow cell, approximately 1400 RU (for the scFv) or 600 RU (for the diabody) of HER2/neu ECD (90 kDa; McCartney, 1994) were coupled to a CM5 sensor chip (Jønsson et al, 1991). Association rates were measured under continuous flow of 5 μl min^{-1} using concentrations ranging from 5.0×10^{-8} to 8.0×10^{-7} M. k_{on} was determined from a plot of [ln (dR/dt)]/t vs concentration (Karlson et al, 1991). Dissociation rates were measured using a constant flow of 25 μl min^{-1} and an scFv or diabody concentration of 1.0×10^{-6} M. Density of HER2/neu ECD

on the sensor chip surface was calculated to be 4.2×10^3 molecules μm^{-2}, assuming 600 RU = 0.007 pmol HER2/neu mm^{-2}.

Cell-surface retention assay

In order to assess the impact of the divalent nature of the C6.5 diabody on its association with cell-bound HER2/neu, an in vitro cell-surface retention assay was performed. For this assay, the C6.5 scFv and diabody were biotinylated using an ImmunoPure NHS-LC-Biotinylation kit (no. 21430, Pierce, Rockford, IL, USA). Twelve micrograms of biotinylated C6.5 scFv or diabody were incubated with 1.2×10^7 HER2/neu-overexpressing SK-OV-3 (HBT 77; American Type Culture Collection, Rockville, MD, USA) cells (Weiner et al, 1993) in a total volume of 0.5 ml of FACS buffer (0.154 M sodium chloride, 10 mM sodium phosphate, 1% bovine serum albumin, 0.1% sodium azide, pH 7.2) for 30 min at room temperature. The cells were centrifuged at 500 g for 5 min at 4°C, washed with 10 ml of ice-cold FACS buffer two times and then resuspended gently in 12 ml of FACS buffer at 37°C. The cell suspensions were then incubated at 37°C with gentle shaking in a water bath. To decrease the rebinding of dissociated biotinylated diabody or scFv to the cells, at 15, 30, 45, 60, 90 and 120 min after commencing the incubation the suspensions were pelleted at 500 g, the supernatants were aspirated and the cells were gently resuspended in fresh FACS buffer (37°C). Immediately after each round of pelleting and resuspension, 0.5-ml aliquots containing 5×10^5 cells were removed in triplicate (i.e. at 0, 15, 30, 45, 60, 90 and 120 min), placed on ice for 5 min and centrifuged at 500 g for 5 min at 4°C. After removing the supernatants from the aliquots, the cells were gently resuspended in 50 μl of ice-cold FACS buffer containing 50 μl of a 1:800 dilution of streptavidin-PE, incubated on ice for 30 min and washed twice with FACS buffer at 4°C. The cells were fixed with 1% paraformaldehyde and the degree of fluorescence was determined by analysis on a FACscan flow cytometer (Becton Dickinson, San Jose, CA, USA) as described (Weiner et al, 1993). k_{off} was calculated assuming first-order kinetic using the formula $F_t = (F_i) \times (e^{-kt})$, where F_t = fluorescence at time t, F_i = fluorescence at time 0 and $k = k_{off}$. $t_{1/2}$ was calculated from k_{off} using $F_t/F_i = 0.5$. Density of HER2/neu on the surface of SK-OV-3 cells was calculated to be 3.2–4.8×10^3 molecules μm^{-2}, assuming a cell diameter of 10 μm and 1.0×10^6 HER2/neu ECD per cell (observations ranged from 1.0×10^6 to 1.5×10^6 in Scatchard assays; unpublished data).
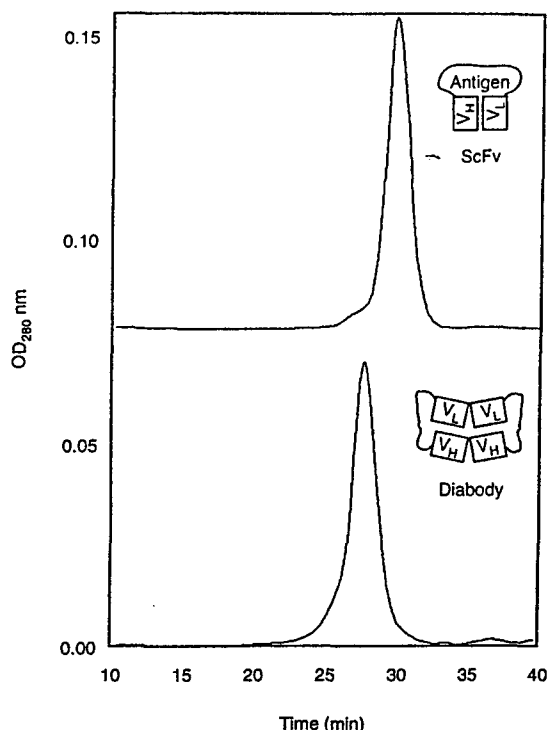
Figure 2 HPLC profiles of the C6.5 diabody and C6.5 scFv. After IMAC purification on a Ni++ agarose column, the C6.5 diabody and scFv were analysed on a Superdex 200 column (Pharmacia). The C6.5 scFv eluted from the Superdex 200 column as a single peak of approximately 25 kDa with minimal evidence of aggregation, while the diabody eluted as a single peak of approximately 50 kDa with no evidence of unassociated monomer

Biodistribution studies

C6.5 diabody and scFv were radiolabelled with iodine-125 using the chloramine T (CT) method (^{125}I/scFv = 1:6; CT/scFv = 1:10) as previously described (Adams et al, 1992, 1993). The quality and

immunoreactivity of the radiopharmaceuticals was evaluated by SDS-PAGE, high-performance liquid chromatography (HPLC) on a Superdex 75 column (Pharmacia) and in a live-cell binding assay as described (Adams et al, 1993). The immunoreactivities of the radiolabelled diabody and scFv monomer were found to be 87.6% and 65.3% respectively. The differences in immunoreactivity most likely reflect the prolonged association of the diabody with its antigen on the cell surface. Six- to eight-week-old CB.17 Icr *scid* mice were obtained from the Fox Chase Cancer Center Laboratory Animal Facility. Then, 2.5×10^6 human ovarian carcinoma SK-OV-3 cells were implanted s.c. on the abdomen of each mouse. When the tumours had achieved a size of 50–200 mg (approximately 8 weeks), Lugol's solution was placed in their drinking water to block thyroid accumulation of radioiodine, and biodistribution studies were initiated. Twenty micrograns (100 μl) of radioiodinated diabody or scFv were administered by i.v. tail vein injection to each mouse. Cohorts of five mice that had received the ^{125}I-C6.5 diabody were sacrificed at 1, 4, 24, 48 and 72 h after injection and a single cohort of five mice that had received the ^{125}I-C6.5 scFv monomer was sacrificed at 24 h after injection. The mean and s.e.m. of retention of each radiopharmaceutical in tissue (%ID g^{-1}) and blood (%ID ml^{-1}) was determined as described (Adams et al, 1993). Calculations of the estimated cumulative localization (AUC) of diabody in tissues and blood were determined using the NCOMP program (Laub, 1996). $t_{1/2}\alpha$ and $t_{1/2}\beta$ were calculated using the Rstrip program (Micromath, Salt Lake City, UT, USA). Significance levels were determined using a Student's t-test on the Statworks program (Cricket Software, Philadelphia, PA, USA).
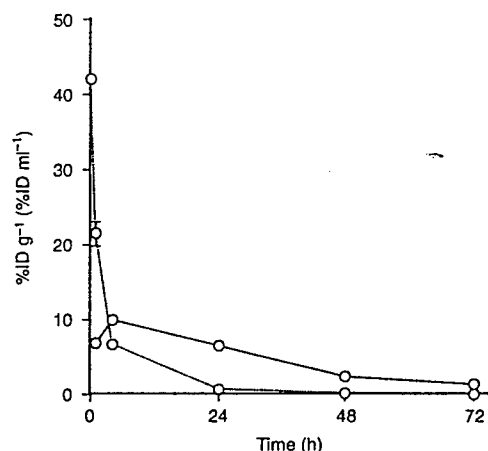
RESULTS

Diabody expression and characterization

The C6.5 diabody and scFv were secreted from *E. coli* grown in shake flasks with typical yields of native protein after IMAC and HPLC purification of approximately 1.0 mg l^{-1} for the diabody and 5 mg l^{-1} for the scFv. The C6.5 scFv eluted from a Superdex 200 column as a single peak of approximately 25 kDa, with minimal
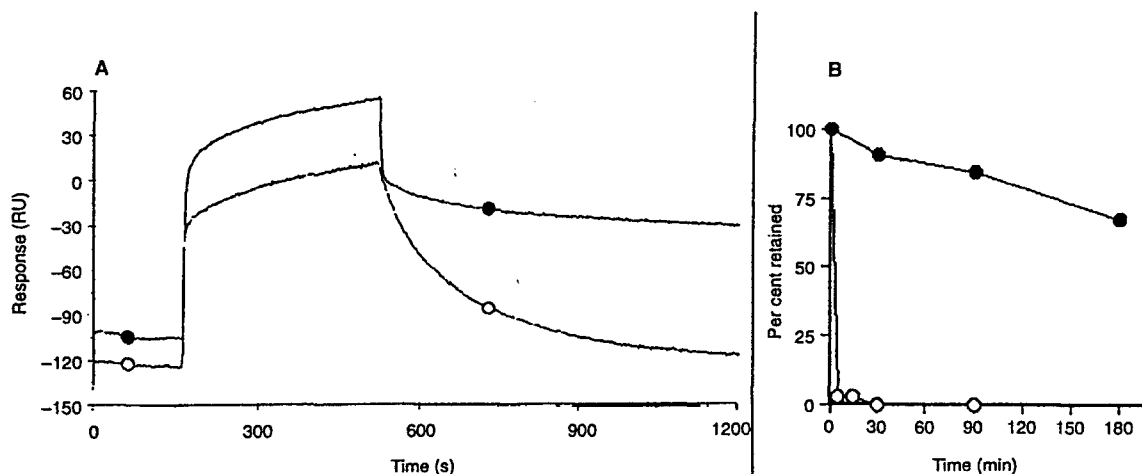
Figure 3 In vitro characterization of the C6.5 diabody. C6.5 diabody and monomeric scFv were evaluated by surface plasmon resonance (BIAcore) as described in Methods. The association and dissociation kinetics are displayed for the diabody (●) and scFv monomer (○) forms or C6.5 (A). In vitro cell-surface retention profiles of biotinylated forms of the C6.5 diabody (●) and C6.5 monomer (○) were determined using SK-OV-3 cells, as described in the text, and the results are displayed; s.e.m.s are less than 2% of the value presented (B)

Figure 4   The in vivo tumour targeting of radioiodinated C6.5 diabody was determined in a biodistribution study performed in SK-OV-3 tumour-bearing *scid* mice. The plotted values represent the mean tumour (●) and blood (○) retentions obtained from six mice per data point. The standard errors are indicated

reduction in k_{off}, which correlated with a retention $t_{1/2}$ of 43 min, compared with 1.8 min for the scFv. In the BIAcore, the HER2/*neu* ECD is chemically coupled to a three dimensional matrix of carboxymethyl dextran, which bears little resemblance to the organization of HER2/*neu* on the cell surface. Therefore, the biological relevance of the increased affinity of the diabody was determined in an in vitro cell surface retention assay using biotinylated C6.5 diabody or scFv and human SK-OV-3 ovarian carcinoma cells overexpressing HER2/*neu*. In this assay, the quantity of biotinylated diabody or scFv retained on the surface of the SK-OV-3 cells over time was determined by flow cytometry. Significantly longer retention of the C6.5 diabody was observed compared with the C6.5 scFv ($t_{1/2}$ scFv = 2.5 min vs $t_{1/2}$ diabody = 5 h; $P < 0.001$) (Figure 2B). The results compare favourably to $t_{1/2}$ values calculated from the k_{off} measured in the BIAcore of 1.8 min for the scFv and 43 min for the diabody. Thus the increase in apparent affinity was much greater on the cell surface than on the carboxymethyldextran surface of the BIAcore, despite the similarities in calculated density of HER2/*neu* sites ($3.2–4.8 \times 10^3$ sites μm^{-2} on the cell surface vs 4.2×10^3 sites μm^{-2} on the sensor chip surface).

Biodistribution assays

The in vivo targeting potential of the C6.5 diabody was assessed in *scid* mice bearing s.c. SK-OV-3 tumours overexpressing the HER2/*neu* antigen. The tumour, blood and organ retention of radioiodinated C6.5 diabody was determined at 1, 4, 24, 48 and 72 h after its i.v. administration. After the injections, the diabody displayed a rapid equilibration phase ($t_{1/2}\alpha = 0.67$ h) and subsequent slower elimination phase ($t_{1/2}\beta = 6.42$ h) from circulation, in a pattern characteristic of small scFv-based molecules (Figure 4). In contrast to the blood retention properties of the diabody, the quantity retained in tumour rose from 6.9% ID g^{-1} at 1 hour post

evidence of dimerization or aggregation (Figure 2A). The C6.5 diabody eluted from a Superdex 200 column as a single peak of approximately 50 kDa, with no evidence of unassociated monomer (Figure 2B). Both migrated under reducing conditions on 12% SDS-PAGE gels as single bands of approximately 27 kDa (data not shown).

The K_d of the C6.5 diabody for HER2/*neu* ECD was determined by surface plasmon resonance in a BIAcore instrument to be 4.0×10^{-10} M ($k_{on} = 6.7 \times 10^5$ M^{-1} s^{-1}; $k_{off} = 2.7 \times 10^{-4}$ s^{-1}) 40-fold lower than the K_d of the C6.5 scFv (1.6×10^{-8} M; $k_{on} = 4.0 \times 10^5$ M^{-1} s^{-1}; $k_{off} = 6.3 \times 10^{-3}$ s^{-1}) (Figure 3A). The decrease in K_d was largely due to

Table 1   Evaluation of [^{125}I]C6.5 diabody in tumour-bearing *scid* mice

	C6.5 diabody							C6.5 scFv
	1 h	**4 h**	**24 h**	**48 h**	**72 h**	**Cumulative AUC**	**Tumour–organ AUC ratio**	**24 h**
Tumour[a]	6.9	10.1	6.5	2.4	1.4	405	–	1.0
Blood[b]	21.5 (0.3)	6.7 (1.5)	0.7 (9.5)	0.1 (22.5)	< 0.1 (24.0)	133	3.0	0.1 (19.1)
Liver	5.7 (1.2)	2.8 (3.6)	0.3 (21.0)	0.1 (22.5)	0.1 (15.0)	137	3.0	< 0.1 (25.4)
Kidneys	16.9 (0.4)	5.2 (1.9)	1.1 (6.0)	0.4 (6.7)	0.3 (4.5)	153	2.6	0.2 (6.3)
Lung	17.0 (0.4)	7.1 (1.4)	0.7 (8.8)	0.1 (19.7)	0.1 (18.2)	57	7.1	0.1 (17.0)
Spleen	4.3 (1.6)	3.5 (3.2)	0.4 (16.1)	0.1 (20.6)	0.1 (17.0)	61	6.6	< 0.1 (24.5)
Heart	13.1 (0.5)	4.7 (2.2)	0.4 (17.5)	0.1 (31.0)	< 0.1 (31.7)	93	4.4	< 0.1 (37.2)
Stomach	4.5 (1.6)	7.9 (1.4)	1.4 (5.7)	0.3 (11.6)	0.3 (6.6)	129	3.1	0.2[c] (6.2)
Intestine	1.6 (4.5)	2.5 (4.6)	0.3 (22.3)	0.1 (33.4)	0.1 (24.7)	37	10.9	< 0.1 (25.8)
Bone	2.3 (3.3)	1.9 (6.0)	0.1 (40.6)	< 0.1 (84.3)	< 0.1 (65.0)	31	13.1	< 0.1[c] (34.2)
Muscle	1.2 (5.8)	1.5 (7.1)	0.2 (34.3)	< 0.1 (44.6)	< 0.1 (53.4)	37	10.9	< 0.1 (49.8)

[a]Expressed as %ID g^{-1} tissue. [b]Expressed as %ID ml^{-1} blood. [c]s.e.m. ≤45%. C.B 17/ICR-scid mice bearing 50- to 200-mg s.c. SK-OV-3 tumours were used in these studies. Cohorts of five mice per time point were given 20 µg of [^{125}I]C6.5 diabody by i.v. injection. The mice were sacrificed at the indicated times and the tumour, blood and normal tissue retention was determined and expressed as a percentage of the injected dose localized per g of tissue (%ID g^{-1}) or per ml of blood (%ID ml^{-1}) as described in Methods. Tumour–organ ratios are presented in parentheses. For each value presented, the s.e.m. was less than 30%, unless otherwise indicated. The cumulative diabody retention (AUC) in each tissue was determined as described and is expressed in arbitrary units to facilitate the determination of the tumour to organ AUC ratios.

injection to a peak of 10.1 %ID g⁻¹ at 4 h post injection and slowly decreased to 6.5% ID g⁻¹ and 1.4% ID g⁻¹ at 24 and 72 h respectively (Table 1 and Figure 4). The retention of the diabody in normal organs reflected the concentration present in blood over the course of the study with the notable exception of the kidneys, which function as the major elimination route for scFv-based reagents (Table 1). The cumulative residence of the radioiodinated diabody in tumour and normal organs, expressed as AUCs, was determined to predict the therapeutic potential for this molecule. Over the course of the study, favourable tumour to organ AUC ratios were observed for a number of organs, including liver (3.0), spleen (6.6), bone (13.1), kidneys (2.6) and blood (3:1) (Table 1). While the activity in the bone marrow compartment is difficult to measure directly, it is routinely estimated based upon the observation that one-fourth of the bone marrow compartment is composed of blood (Siegel et al, 1990). As HER2/*neu* is not expressed on cells in the marrow, the diabody will not specifically bind to marrow, just as it does not bind to other tissues lacking HER2/*neu* (e.g. liver, spleen and muscle). Therefore, the radioiodinated diabody present in the bone marrow compartment can be solely attributed to that present in the blood portion of the bone marrow. Accordingly, the tumour to bone marrow ratio was estimated as 12:1 (25% of the tumour–blood ratio).

The biodistribution of the [^{125}I]C6.5 scFv monomer was performed at 24 h after administration for comparative purposes and was found to be virtually identical to that previously reported for this and other scFv monomers of similar affinity, with 1.0 % ID g⁻¹ retained in tumour, 0.04 % ID g⁻¹ in liver and 0.05 % ID ml⁻¹ in blood (Table 1) (Colcher et al, 1990; Milenic et al, 1991; Adams et al, 1993; Schier et al, 1995). This clearly demonstrated the significantly increased tumour retention ($P = 0.00043$) conferred by the diabody format. The prolonged blood retention of the larger diabody molecule may also account for some of the increased tumour retention. This is evidenced by the 24-h tumour–blood ratios of about 9:1 for the diabody and 20:1 for the monomer.

DISCUSSION

Here we describe the production and in vitro and in vivo properties of the C6.5 diabody molecule specific for HER2/*neu*. The C6.5 diabody was expressed and purified in high yield from *E. coli* as native protein without refolding. Compared with the scFv from which it was derived, the diabody exhibited a significantly lower K_d and slower k_{off} from HER2/*neu* that was either immobilized on a BIAcore sensor chip or as expressed on the surface of tumour cells. In vivo, radioiodinated C6.5 diabody displayed an excellent balance of quantitative tumour deposition and specificity. Peak tumour values of 10 %ID g⁻¹ were observed at 4 h after intravenous administration and persisted through 24 h (6.5 %ID g⁻¹) and 72 h (1.2 %ID g⁻¹) post injection. In contrast, the diabody was rapidly cleared from the circulation and antigen-negative organs, as its molecular weight (50 kDa) is less than the renal threshold. As a result, significantly more diabody was retained in tumour than in any other organ at all but the earliest time points studied. This yielded tumour–normal organ AUCs of 3:0 (tumor–blood) to 13.1:1 (tumour–bone). Furthermore, as we and others have previously demonstrated, antibody-based molecules with sizes beneath the renal threshold for first-pass clearance are typically eliminated in a biphasic manner, with a rapid initial equilibration phase and a slower elimination phase (reviewed in Huston et al, 1996). This suggests that the sampling times used in this study may have

exaggerated the blood AUC value for the interval between 4 and 24 h. Thus, it is likely that the inclusion of additional sampling times (i.e. 6, 12 and 16 h post injection) would reveal lower blood retentions and hence, more specific tumour localization. As the C6.5 diabody was developed from a phage display-derived scFv, a C6.5 IgG molecule was not available for a direct comparison between these two divalent structures. However, a reasonable comparison can be made using an IgG molecule specific for a different epitope on the HER2/*neu* antigen. We have previously reported on the distribution of 741F8 IgG, which, like many other monoclonal antibodies targeting cell-surface tumour-associated antigens, exhibits a high degree of tumour uptake (e.g. 20 %ID g⁻¹) with very poor targeting specificity (tumour–blood ratios ≤ 1:1) (Weiner et al, 1995). Therefore, even though the degree of tumour retention observed with the C6.5 diabody was less than that observed with anti-HER2/*neu* IgG, the increased targeting specificity associated with the diabody format results in an advantage.

Compared with the scFv, the increased tumour deposition of the diabody could result from its increased size or increase in apparent affinity (avidity). The increased size of the C6.5 diabody led to a slower redistribution and elimination $t_{1/2}$ than was observed for the C6.5 scFv. This leads to higher blood levels and prolongation of the concentration gradient for diffusion from blood into tumour. However, Fab are of similar size and have similar pharmacokinetics, but do not provide as great an increment in quantitative tumour retention compared with scFv (Milenic et al, 1991; Adams et al, 1993). Thus, size alone is unlikely to account for the increased tumour deposition and retention of the diabody, which instead must be at least partly due to an increase in apparent affinity resulting from avidity. A priori, it was unclear to what extent an increase in apparent affinity would occur with the divalent diabody molecule. The Fab arms of the IgG molecule are extremely flexible, because of the hinge (Ferencik, 1993). In contrast, the two binding heads of the diabody are oriented 180° apart in a rigid configuration (Perisic et al, 1994). Thus, the extent to which the diabody could engage two antigens simultaneously, particularly on the cell surface, was unclear. As determined using surface plasmon resonance in a BIAcore, the apparent affinity of the diabody is 40-fold higher than the scFv, largely because of a 40-fold reduction in k_{off}. The dissociation of diabody from the cell surface was sevenfold slower than observed on the BIAcore, with a k_{off} approximately 280-fold slower than the scFv. As the calculated antigen density on the BIAcore sensor chip surface and the cell surface are approximately the same, these differences may result from the greater mobility of HER2/*neu* ECD in the cell membrane, leading to bivalent binding without steric strain.

Monoclonal antibody (MAb)-based radioimmunotherapy has shown notable promise in the treatment of haematological malignancies (Kaminski et al, 1993; Press et al, 1993), but progress in the therapy of solid tumours has been hindered by a number of factors dictated by tumour physiology (Jain, 1990). First, the disordered vasculature of solid tumours leads to a heterogeneous intratumoral distribution of MAb. Second, the paucity of draining lymphatics in tumours results in elevated hydrostatic pressure, limiting the diffusion of large molecules such as IgG to 100 μm in 1 h, 1 mm in about 2 days and 1 cm in about 7–8 months. To obtain sufficient tumour localization for radiolabelled MAb to provide therapeutic effects, the MAb must remain in circulation long enough to diffuse from blood into tumour. At the same time, the radiolabelled MAb must be eliminated from circulation rapidly enough to diminish normal organ retention and prevent unacceptable toxicities. To

achieve successful RAIT, a proper balance must be established between these competing requirements. We hypothesized that an appropriate balance could be accomplished by using small, high-affinity, multivalent, antibody-based molecules. Decreasing the size of the molecule increases both its diffusion rate into tumour (Jain, 1990) and its rate of elimination from circulation, thus enhancing both the degree of tumour penetration and the specificity of tumour retention. While the optimal size for an antibody-based construct has yet to be identified, we believe it will fall below the renal threshold for first-pass clearance (about 65 kDa). When administered by a continuous i.v. infusion, such molecules could be maintained at steady-state levels in circulation, and controlled gradients from blood into tumour could be established. This would facilitate deep penetration into tumour and highly specific tumour retention when the molecules are rapidly eliminated from circulation upon the termination of the infusion. A variety of molecular structures that span a wide range of sizes are available. These include 80-kDa (scFv-CH3)$_2$ minibodies (Hu Shizhen et al, 1996), 50-kDa diabodies (Holliger et al, 1993), 27-kDa scFv (Bird et al, 1988; Huston et al, 1988) and individual 12- to 13-kDa V_H or V_L chains (Ward et al, 1989). While the smallest molecules will be capable of the greatest diffusion into solid tumours, their administration will require careful management to maintain the blood concentrations required to permit diffusion through tumour. Increasing the functional affinity may help 'trap' the scFv that diffuses into tumour, localizing it long enough to facilitate therapeutic applications. This can be accomplished by manipulating the intrinsic affinity properties (Schier et al, 1996) or through the creation of multivalent binding proteins. While Weinstein has hypothesized that the diffusion of high-affinity MAb into tumour is hindered by binding to antigen-bearing cells close to blood vessels (Fujimori et al, 1989; Juweid et al, 1992), this may be overcome by enhancing the diffusion gradient from blood into tumour through the administration of large doses. Finally, the potential of engineered antibody-based proteins to target tumours in humans in a highly specific manner was recently demonstrated using radio-immunoimaging performed by Begent et al (1996). Given that successful tumour localization in the above study was achieved with a small, monovalant scFv, it is our belief that the larger, divalant diabody molecule used here will also exhibit impressive tumour targeting in patients.

Of the divalent scFv-based molecules produced to date, before this study, reports of in vivo assays only exist for the (scFv')$_2$ and the minibody. Previously, we have shown that the tumour retention of a 20-μg dose of the anti-HER2/*neu* 741F8 (scFv')$_2$ in *scid* mice bearing relevant tumours is twice that seen with 741F8 scFv monomer (Adams et al, 1993). While the specificity of tumour retention at 24 h post injection was very high, as evidenced by tumour–blood and tumour–muscle ratios of 10:1 and 75:1, respectively, the quantity of (scFv')$_2$ retained in tumour (1.6 %ID g^{-1}) was insufficient to mediate therapeutic effects or predict for therapeutic dosimetry in tumours. Hu Shi-Zhen et al (1996) have recently reported excellent selective tumour retention after the administration of small quantities (0.1–0.2 μg per mouse, or 0.005–0.01 μg/g^{-1} body weight) of anti-CEA (scFv-C$_H$3)$_2$ minibodies to tumour-bearing athymic mice, with average 24-h retentions of 29 %ID g^{-1} and 8 %ID g^{-1} in tumour with flex and LD minibodies respectively. While the mass of the C6.5 diabody (50 kDa) lies just below the renal threshold for first-pass clearance, the two minibody species have molecular weights of approximately 80 kDa and are above the threshold. This is evidenced by

the faster clearance of the diabody from circulation (0.7 %ID ml^{-1} vs 2.1 %ID ml,$^{-1}$ respectively, for the diabody and minibodies at 24 h post injection), which probably leads to a lower cumulative blood, and hence marrow, exposure for the diabody. However, the greater peak tumour retention of the minibody leads to similar tumour–blood AUCs for both molecules. Clearly, the parallel evaluation of identical doses of a series of reagents (i.e. scFv, (scFv')$_2$, diabody and minibody) with identical specificity is desirable to definitively address the role of size on the in vivo tumour-targeting properties of these recombinant antibody-based molecules.

The cumulative retention (AUC) of C6.5 diabody in tumour and normal tissues was calculated to predict the therapeutic potential of diabodies as vehicles for RAIT. RAIT efficacy is dependent upon the delivery of lethal doses of radiation to tumour without exceeding the doses tolerated by the bone marrow (200–300 cGy) and organs involved in the catabolism of the radiopharmaceutical, such as the kidneys (1500 cGy) and the liver (4000 cGy) (Bentel et al, 1989). In this study with the C6.5 diabody, we calculated tumour to organ ratios ranging from 3:1 to 13:1. The tumour–bone marrow estimate of 12:1 and tumour–kidney value of 2.6:1 would permit the delivery of approximately 4000 cGy to tumour at a marrow dose of 250 cGy. This represents a significant improvement over results in preclinical models observed with other antibody-based molecules, including scFv (Adams et al, unpublished results), (scFv')$_2$ (Weiner et al, 1995), Fab (Yorke et al, 1991), F(ab')$_2$ (Stein et al, 1991, 1994) and IgG (Stein et al, 1991; Molthoff et al, 1991, 1992). While the predicted tumour–blood AUCs for Flex minibodies are similar to those reported here for the C6.5 diabody, the smaller diabody structure may confer an advantage when penetration of large solid tumours is required.

As the divalent binding of a diabody molecule to antigen on the surface of a tumour cell molecule is dependent upon both the density of the antigen and its orientation, it is likely that such binding would only occur when the antigen density is high. While the C6.5 diabody remains bound to the tumour cells in vitro (Figure 3B) and is retained in tumour in vivo (Figure 4) significantly longer than is its monomeric scFv form, it is likely that diabody bound to normal tissue expressing low concentrations of HER2/*neu* would bind in a monovalent manner and exhibit the rapid dissociation kinetics characteristic of the C6.5 scFv. To confirm this hypothesis, the in vitro and in vivo binding profiles of C6.5 diabody and C6.5 scFv require evaluation in tumour cell lines and tumours with a wide range of HER2/*neu* expression.

If the prolonged retention of the C6.5 diabody on the surface of cells and in tumours overexpressing HER2/*neu* is mediated by divalent binding, it may exert a direct biological impact on these cells. Homodimerization of HER2/*neu* or heterodimerization of HER2/*neu* with c-erbB-3 or c-erbB-4 has recently been found to be required for signal transduction after the binding of heregulin to c-erbB-3 or c-erbB-4 (Earp et al, 1995; Wallasch et al, 1995). The possibility that divalent binding of two HER2/*neu* molecules by C6.5 diabody facilitates the homodimerization of HER2/*neu* with subsequent signal transduction is intriguing. Alternatively, it is possible that cytostatic effects could be triggered by the immobilization of HER2/*neu* on the cell surface to prevent the homodimerization of the molecule's transmembrane region. Either of these mechanisms may be responsible for reports of synergistic effects between some anti-HER2/*neu* monoclonal antibodies and chemotherapeutic agents, such as taxol or cisplatin (Hancock et al, 1991). Accordingly, the potential of the C6.5 diabody to dimerize HER2/*neu*, trigger signal transduction and inhibit tumour cell

growth in the presence of chemotherapeutic agents has been studied. However, when the C6.5 diabody has been assayed for growth inhibition potential by in vitro MTT [3-(4,5-dimethylthi-azol-2-yl)-2,5-diphenyltetrazolium bromide] incorporation assays, concentrations of up to 10 μg ml⁻¹ for 7 days do not significantly inhibit the growth of SK-OV-3 cells overexpressing the HER2/*neu* antigen (unpublished results). Accordingly, it is probable that the C6.5 diabody by itself is not capable of exerting cytostatic effects.

Continued improvements in antibody engineering have led to increasingly sophisticated structures that address impediments to successful tumour targeting. The C6.5 diabody may be an effective targeting vehicle for RAIT. In addition, this molecule may provide a useful platform for the creation of affinity mutants with slower k_{off} rates or for the creation of fusion proteins containing other antibodies, cytokines, chemotactic factors or toxins.

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Appendix 4

EFFICIENT CONSTRUCTION OF A LARGE NON-IMMUNE PHAGE ANTIBODY LIBRARY: THE PRODUCTION OF PANELS OF HIGH AFFINITY HUMAN SINGLE CHAIN ANTIBODIES TO PROTEIN ANTIGENS

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Abbreviations: ActRI, activin receptor type I; ActRII, activin receptor type II; AMP, ampicillin; BMP, bone morphogenetic protein; BMPRI, BMP receptor type I; BoNT, botulinum neurotoxin; bp, basepairs; ECD, extracellular domain ; CDR, complementarity determining region; EB, elementary body of *chlamydia tradomatis*; FGFR, fibroblast growth factor receptor; MBP, maltose binding protein; RTPCR, reverse transcription PCR; scFv, single chain Fv fragment; VEGF, vascular endothelial growth factor; 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.

Abstract:

A large library (6.7×10^9 members) of phage displayed human single-chain Fv antibodies was generated by improving and increasing the efficiency of library construction. 14 different protein antigens were used to affinity select antibodies from this library. With each antigen a panel of antibodies was isolated that on average contained 8.7 different specific antibodies. Antibody affinity was comparable to antibodies from the secondary murine immune response with some affinities below 1 nM. Four different antibodies recognizing ErbB2 had affinities ranging from 220 pM to 4 nM. Antibodies derived from the library proved to be useful in immunoassays such as ELISA and immunoflourescence. For example, antibodies to *Chlamydia trachomatis* elementary bodies stained *Chlamydia* infected cells but not uninfected cells. These results demonstrate that phage antibody libraries are ideally suited for the rapid production of panels of high affinity monoclonal antibodies to a wide variety of protein antigens. Such libraries should prove particularly useful for the generation of antibody reagents to study the function of genes identified by genome projects.

INTRODUCTION:

Antibodies that bind with high specificity and high affinity to a target molecule are essential tools for biological research. These reagents have proven invaluble for : 1) detecting and quantitating levels of gene expression; 2) determining the subcellular, cellular and tissue location of gene expression; and for 3) identifying the molecules interacting with a gene product, for example by immunoprecipitation.

Numerous new applications for basic research, as well as clinical use, have resulted from the development of recombinant antibodies constructed from immunoglobulin variable (V) region genes (1-3). Single chain Fv antibodies (scFv) have proven particularly useful. scFv consist of the antigen binding domains of immunoglobulin heavy (V_H) and light (V_L) chain regions connected by a flexible peptide linker (4) all encoded by a single gene. The single gene design of scFv simplifies the construction of fusion proteins such as cancer immmunotoxins (5), and facilitates intracellular expression in eukaryotic cells to achieve phenotypic knockout of antigen function. The intracellular expression of antibodies is proving to be an effective new strategy for studying the function of specific proteins *in vivo* where conventional genetic approaches are not feasible (6-8).

Genome projects have led to an increasing rate of gene discovery and an accelerating need for antibodies to study gene expression and function. Until recently, the production of monoclonal antibodies for such application were generated using hybridoma technology, a slow and cumbersome process. Separate immunizations are required for each antigen and the cell fusion process required to generate hybridomas is laborious and inefficient. In addition, production of antibodies to antigens conserved between species is difficult and antibodies from hybridomas are murine and hence immunogenic if used therapeutically.

Recently, using antibody phage display it has proven possible to overcome these limitations (1-3, 9). For phage display, the antigen binding regions of V_H and V_L genes are cloned and used to construct scFv (or Fab) gene repertoires. A phage antibody library is created by cloning these

repertoires as fusion proteins with a minor coat protein of bacteriophage (the gene 3 protein) (10-12). Each resulting phage has a functional antibody protein on its surface and contains the gene encoding the antibody incorporated into the phage genome. Particular phage antibodies that specifically bind to proteins and small molecules can be separated from non-binding phage antibodies with affinity chromatography techniques (12-15). This strategy requires no immunization, the antibody genes are cloned and the antibody fragments express well in *E. coli*. The number and affinity of the antibodies generated to a particular antigen is a function of library size and diversity, with larger libraries yielding a greater number of high affinity antibodies (14, 15). Unfortunately, the construction of large phage displayed antibody libraries has remained difficult. If such libraries are to be a common tool of life scientists the efficient production of these reagents must become routine, especially since library diversity and utility are lost upon library reamplifcation.

In this report, we describe a strategy to optimize phage display antibody library construction. By employing this strategy, a very large phage-displayed single-chain antibody library consisting of 6.7×10^9 members was produced and then used to isolate panels of antibodies to 14 different protein antigens. Analysis of antibody-antigen interactions revealed high affinity binding with K_ds ranging between 5nM and 220pM.

METHODS:

Construction of heavy chain variable region (V_H) library. Total RNA was prepared from 3 different human spleen samples and 2 different samples of human peripheral blood lymphocytes. cDNA was synthesized from total RNA primed with HuIgMFOR (12). V_H gene repertoires were amplified from the cDNA using Vent DNA polymerase (New England Biolabs) in combination with the HuIgMFOR primer and an equimolar mixture of HuVHBACK primers (12). PCR products were agarose gel purified, extracted from the gel using Geneclean (Bio101) and reamplified to append *Nco*I and *Not*I restriction sites using Tth DNA polymerase (Epicentre) and an equimolar mixture of the

HuVHBACKSfi primers (that contain a *Nco*I restriction site for cloning) and the HuCMForNot primer (5'-GAGTCATTCTCGACTTGCGGCCGCTGGAAGAGGCACGTTCTTTTCTTT-3')(12). The PCR products were cut with restriction enzymes *Nco*I and *Not*I. The resulting DNA fragments were agarose gel purified, ligated into the plamid pCITE3A (Novagen) cut with restriction enzymes*Nco*I and *Not*I and the ligated DNA electroporated into the *E.coli* strain TG1. A new library of V_H genes containing 2.3×10^8 members was generated from the products of 7 ligation reactions and 15 electroporations. The resulting library was termed pCITEV$_H$. Cloning efficiency and library diversity was determined by PCR screening (12, 16). The pCITE3A plasmid was used to create the V_H gene repertoire due to the presence of unique sequences for specific PCR amplification that surround the cloning sites of this plasmid. These unique sequences allow the specific amplification of only newly generated V_H genes for scFv assembly and avoid the unwanted amplification of existing V_H genes that may be present as comtamints. This strategy is advantageous for amplification of the V_H genes and also the subsequent amplification of scFv genes assembled from the new V_H genes. Although we chose the pCITE3A plasmid for production of our V_H gene repertoire, any plasmid that contains the proper restriction sites for cloning and unique sequences for specific PCR amplification would have been suitable for this purpose.

Construction of scFv library. The V_H gene repertoire was PCR amplified from the pCITE-V$_H$ library using 300ng of library plasmid DNA as a template, Vent DNA polymerase, the CITE3 primer (5'-GATCTGATCTGGGGCCTCGGTGC-3') and an equimolar mixture of HuJ$_H$ primers (12). The V_L genes for scFv assembly were obtained from a previously constructed scFv phage antibody library (12). The V_L gene repertoire, including DNA encoding the scFv peptide linker (G4S)3 (4), was amplified from 300ng of library_plasmid DNA using Vent DNA polymerase, the Gene3 primer (5'-GCAAGCCCAATAGGAACCCATGTACCG-3') and an equimolar mixture of RHuJ$_H$ primers (12). The amplified V_H and V_L genes were agarose gel purified and spliced together with overlap extension PCR to create a scFv gene repertoire (11). To accurately join V_H and V_L gene repertoires together

with overlap extension PCR, the DNA fragments to be joined must have blunt ends. Therefore, the proofreading DNA polymerase Vent was used to generate DNA fragments for scFv assembly. For all subsequent PCR steps of library construction Tth DNA polymerase was found to be the optimal enzyme for DNA amplification. The V_H and V_L gene repertoires were splice together in PCR reactions containing 100ng of the V_H and V_L gene repertoire and 2 units Tth DNA polymerase. These reactions were cycled 8 times (95°C, 2 min., 55°C, 1 min. and 72°C, 3 min.) to join the fragments, then the CITE3 and Gene3 primers were added and the reaction cycled 30 times (94°C, 1 minute; 55°C, 1 minutes; and 72°C, 3 minutes) to amplify the assembled scFv genes. The scFv genes were cut with restriction enzymes *Nco*I and *Not*I, agarose gel purified and ligated into the plasmid pHEN-1 (17) cut with *Nco*I and *Not*I. The ligated DNA was electroporated into *E.coli* TG1 cells.

Proteins. The extracellular domains of the *Xenopus* activin receptor type I (A. Suzuki and N. Ueno unpublished), activin receptor type II (18), BMP receptor type I (19, 20) and FGF receptor (21) were cloned into pMAL expression plasmids as fusions with maltose binding protein (MBP), expressed and purified from *E.coli*. (New England Biolabs). Neuronal bungarotoxin was purchased from Biotoxins. *Clostridia botulinum* neurotoxin type A (BoNT/A) was provided by Ray Stevens and BoNT/B, C and E by Theresa Smith. BoNT/A C-fragment was purchased from Ophidian. Human ErbB-2 ECD was provided by James Huston (22), human cytochrome b5 by Lucy Waskell and human VEGF by James Hoeffler.

Selection of phage antibodies. Phagemid particles were rescued from the library, as described (23) except that the procedure was scaled up to two liters of culture media. Specific phage-displayed scFv were affinity selected using proteins absorbed to Immunotubes (Nunc) (12). For selections with MBP fusion proteins, phage were preincubated with 50μg of purified MBP to deplete the library of antibodies to MBP. For selection of scFv to the Erb-B2 ECD, Immunotube selection was alternated with selection using decreasing concentrations of biotinylated Erb-B2 ECD and capture of bound phage using streptavidin paramagnetic beads (23). For selection of scFv binding *Chlamydia*

antigens, Immunotubes were coated overnight at room temperature with 1ml of *Chlamydia trachomatis* strain L2/434/Bu elementary bodies (EBs) at a concentration of 0.1mg/ml (in PBS) purified from a suspension culture of L929 cells (24). Phage eluted from each selection were used to infect *E.coli* TG1 cells. Phage particles were rescued from the cells and used for the subsequent round of antigen selection. The rescue-selection-plating cycle was repeated 3 to 4 times, after which individual clones were analyzed for specific antigen binding by ELISA (enzyme linked immunosorbent assay).

Antibody binding specificity. The binding specificity of all scFv was determined by ELISA with the target antigen and compared to at least nine other proteins (12). The number of unique scFv was estimated by PCR fingerprinting with the restriction enzyme *Bst*NI and confirmed by DNA sequencing of the scFv gene (12, 16). Putative V_H and V_L germline gene segment derivation was determined by alignment to the VBASE sequence directory (25).

scFv purification and affinity measurements. For purification, scFv genes were subcloned, expressed and purified to homogeneity (26). scFv dissociation equilibrium constants (K_d) were calculated from the association (k_{on}) and dissociation (k_{off}) rate constants determined using surface plasmon resonance in a BIAcore (23, 27).

Fluorescent cell staining. Monolayers of HeLa 229 cells were grown on coverslips in 24 well cell culture plates. 200μl of *Chlamydia trachomatis* EBs at 8×10^6 inclusion forming units/μl were used to infect the monolayers (28). The infected cells were incubated for 48 hrs at 37°C, washed with PBS and fixed with 100% methanol for 10 minutes. Purified scFv (50μg/ml) was incubated with fixed cells for 1hr at room temperature. Binding was detected with the 9E10 monoclonal antibody that recognizes the c-myc epitope present in the scFv (29) (1μg/ml) followed by FITC-conjugated anti-mouse Fc (Zymed). Cells were counterstained with Evans Blue and visualized using a fluorescent microscope.

RESULTS:

Library construction. A very large phage antibody library was created for the routine isolation of high affinity scFv antibodies to any target protein. A strategy was employed to optimize library construction by increasing the efficiency of scFv gene assembly and increasing the efficiency of cloning scFv genes (Fig. 1). First, scFv were assembled from separate V_H and V_L gene repertoires contained in separate plasmid vectors. A new library of V_H genes containing 2.3×10^8 members was specifically created for scFv assembly. An existing scFv repertoire containing 3.0×10^7 members was used as the source of V_L genes (Fig. 1A). The use of cloned libraries as a source of V-genes provided a stable and limitless supply of material for scFv assembly. Previously, scFv gene repertoires were directly assembled from V_H and V_L RTPCR products and linker DNA (12). With this previous approach the quantity of V-genes available for library construction was limited by the amount of RNA available and the efficiency of the RTPCR reactions. Second, the efficiency of scFv assembly was increased by exploiting the presence of the DNA encoding the peptide $(G_4S)_3$ linker located at the 5' end of the V_L library (Fig. 1B). Using V_L genes already fused to the peptide linker allowed us to construct scFv from only 2 DNA fragments. Previously, scFv gene repertoires were assembled from 3 separate DNA fragments consisting of V_H and V_L gene repertoires and linker DNA (12). Third, the primers used to amplify the assembled V_H and V_L gene repertoires annealed to sequences approximately 200 bp upstream of the V_H genes and to sequences approximately 200 bp downstream of the V_L genes. The presence of these long sequence overhangs on the correctly assembled scFv genes ensured efficient cutting with the restriction enzymes *NcoI* and *NotI* that were used for scFv gene cloning (Fig. 1C).

Using these three modifications a repertoire of scFv genes was efficiently assembled and cloned to create a phage antibody library containing 6.7×10^9 members. This library was generated from the products of only 12 ligation reactions and 36 electroporations. DNA sequencing of the V-

genes from 36 randomly chosen scFv revealed 36 unique sequences and a relatively random distribution of V_H CDR3 length of between 5 and 18 residues (Fig. 2). There was, however, bias in V-gene usage, with both over representation of specific V-gene families (V_H3, $V_\kappa 1$ and $V_\lambda 3$) and V-genes (DP-47, DPL 16) (Fig. 2). This bias partially reflects differential V-gene usage observed in the human B-cell repertoire (30-33) but may also be partly due to differences in the efficiency of PCR primer annealing to the different V-genes. Previous work indicates that more diverse repertoires could be created by using V_H and V_κ gene family specific primers individually rather than pooled for construction of the V-gene repertoires (34).

Selection and characterization of antigen specific scFv. Antibodies from the phage antibody library were affinity selected using 13 different purified protein antigens and *Chlamydia trachomatis* elementary bodies (EB) (Table 1). These proteins were from a variety of species, including human. Given our interest in developmental biology, the proteins included the extracellular domains of 4 different *Xenopus* growth factor receptors expressed as maltose binding protein (MBP) fusions in *E. coli* (the activin receptor types I and II, the bone morphogenic protein receptor type I, and the fibroblast growth factor receptor) (19-21). After at least three rounds of selection binding specificity of individual scFv were determined by ELISA. A high percentage of the clones analyzed specifically bound the antigen used for selection (Table 1, second column). To determine the number of different scFv that recognized each antigen, ELISA positive clones were characterized first by DNA fingerprinting (12, 16) and then sequencing (23). This characterization revealed that an average of 8.7 different antibodies were generated to each protein antigen, with the number of scFv ranging from 3 to 15 (Table 1). Since only a small number of clones were analyzed, it is likely that screening of more clones from each selection would yield additional antibodies.

The binding of scFv to antigens was highly specific. For example, serotype specific scFv were isolated against each of the four different types of BoNT, despite 32-59% sequence homology between the toxins (Fig. 3). Another example of scFv specificity is shown in Fig. 4, where a *Chlamydia*

trachomatis specific scFv stains *C. trachomatis* elementary bodies within infected cells while neighboring uninfected cells remain unstained.

V-gene derivation of binding scFv was diverse (Fig 2). V_H genes were derived from 3 of the 6 V_H gene families (1, 3, and 5) and from 26 different germline genes. V_L genes were derived from 3 of the 6 V_κ gene families (1, 3 and 4) and 11 different V_κ germline genes and from 3 of the 9 V_λ gene families (1, 2 and 3) and 9 different V_λ germline genes. Despite the diversity, there was a bias seen in the V-gene usage. V_H genes were largely derived from the VH3 family, particularly DP46 and DP47. V_κ genes were most frequently derived from the $V_\kappa 1$ family while V_λ genes were most frequently derived from the $V_\lambda 3$ family, especially DPL-16. This bias partially reflects the greater frequency of certain V genes in the B-cell repertoire (30-33) and also in the unselected library (for example DP-47 and DPL-16). Differential V-gene usage may also reflect expression biases of *E. coli*. The number of sequenced V-genes from previous non-immune phage antibody libraries is small (approximately 30) but a similar bias in V-gene usage is observed (12, 35, 36). V_H CDR length of selected clones was not as evenly distributed as in the unselected clones (Fig. 2) with the majority of lengths between 7 and 15 amino acids. A similar peak is seen in V_H CDR3 length of antibodies generated *in vivo* (37).

Affinity of selected antibodies. The antibody antigen binding affinities were measured for several of the anti-ErbB-2 and anti-BoNT/A scFv. The genes of four anti-ErbB-2 scFv and four anti-BoNT/A scFv were subcloned into a plasmid to add a hexahistidine tag, then expressed and purified from *E.coli*. The dissociation equilibrium constants (K_d), of purified soluble anti-ErbB-2 and anti-BoNT/A scFv was calculated from association and dissociation rate constants measured using surface plasmon resonance (Table 2) (23, 27). The K_d of the antibodies ranged from 220pM to 4nM for anti-ErbB-2 scFv and 38nM to 71nM for anti-BoNT/A scFv. The affinity of the anti-ErbB-2 scFv B7A is the highest observed for any antibodies isolated from non-immune phage antibody libraries (14, 15).

The affinities of the isolated scFv are also comparable to affinities of monoclonal antibodies derived from the secondary immune response (38).

The different K_ds observed for scFv that bind ErbB-2 and BoNT/A is probably a consequence of the different selection conditions used to isolate each panel of antibodies. ErbB-2 antibodies were selected with decreasing concentrations of soluble antigen captured with magnetic beads alternated with selections using immobilized antigen. The use of soluble antigen is a more efficient method for controlling and decreasing the concentration of antigen used for selection and for isolating scFv with higher affinity (23, 39). Therefore, reselection of antibodies using decreasing concentrations of BoNT/A would likely lead to the isolation of antibodies with higher binding affinity.

DISCUSSION:

A very large scFv phage antibody library was efficiently generated and its use as a resource for the production of antibodies was extensively evaluated. Using a number of different criteria, the results validate our methods for constructing large libraries of this type and validate the use of these libraries as a resource for the rapid production of antibodies (Table 1). First, using 14 different proteins for affinity selection, specific antibodies were successfully generated to each of these antigens (Table 1). Second, a high percentage of the antibodies that resulted from affinity selection specifically recognize antigen (Table 1). Third, multiple different antibodies were produced to each antigen (Table 1). Fourth, the binding affinities of the antibodies isolated were comparable to those of monoclonal antibodies from the secondary murine immune response (Table 2). In addition, these antibody/antigen binding affinities are the highest reported for antibodies from non-immune phage antibody libraries (12, 14, 15). Fifth, isolated scFv served as functional reagents in a number of different immunoassays including ELISA, immunoflourescence (Fig. 3 and 4) Western blotting, epitope mapping and immunoprecipitation (data not shown).

Non-immune phage antibody libraries can be constructed as either scFv or Fab antibody fragments and from either V-genes rearranged *in vivo* or synthesized *in vitro*. The scFv format was

chosen for this library as the expression levels in *E.coli* are typically higher than Fab. This results in more efficient antibody display on phage and more efficient production of native antibody fragments for use. V-genes rearranged *in vivo* were used for library construction to eliminate the need for cloning the individual gene segments necessary for *in vitro* V-gene synthesis. In addition, use of immunoglobulin mRNA as the source of V-genes ensures that close to 100% of the gene sequences will be functionally rearranged with open reading frames (sequencing results from this work and (34). Fewer V-genes will have an open reading frame when constructed from synthetic oligonucleotides. Furthermore, V-genes rearranged *in vivo* have V$_H$ CDR3s largely derived from the D-gene segments. These genes are not of random sequence but encode amino acids with a propensity for loop formation (40). In contrast, synthetic CDR3s consist of random sequence and thus may be less likely to fold properly or produce usefully shaped binding pockets.

The number and affinities of antibodies produced from this library compare favorably to results from the limited number of phage antibody libraries previously described (Table 3). A comparison of non-immune libraries illustrates the importance of library size and also suggests that to date, the most useful libraries are those in the scFv format constructed from V-genes rearranged *in vivo*.

Non-immune phage antibody libraries are already being used as a source of diagnostic and therapeutic antibodies. It is likely that their greatest utility, however, may lie in the laboratory. New genes are rapidly being identified by the genome projects and the next generation of experiments will shift to elucidating the function of the protein products encoded by these newly identified genes (41). The production of antibodies with phage-displayed libraries is ideally suited for the large-scale determination of protein function. For example, once a gene has been sequenced, the protein(s) that it encodes can be overexpressed and then used to rapidly select phage displayed antibodies. The resulting antibodies would provide immunological reagents for protein characterization. In addition, the production of antibodies with phage display also provides access to the genes that encode specific

antibodies. These antibody genes can be used to express antibody proteins within cells to block the function of specific proteins *in vivo* and elucidate protein function (6-8).

In summary, the steps of phage antibody library construction have been optimized to facilitate the rapid and efficient construction of large phage antibody libraries. With this current library we obtain panels of high affinity antibodies to a wide array of antigens. The approach used puts this technique within reach of laboratories skilled in molecular biology. Subsequent uses for these libraries will be limited only by the investigators imagination.

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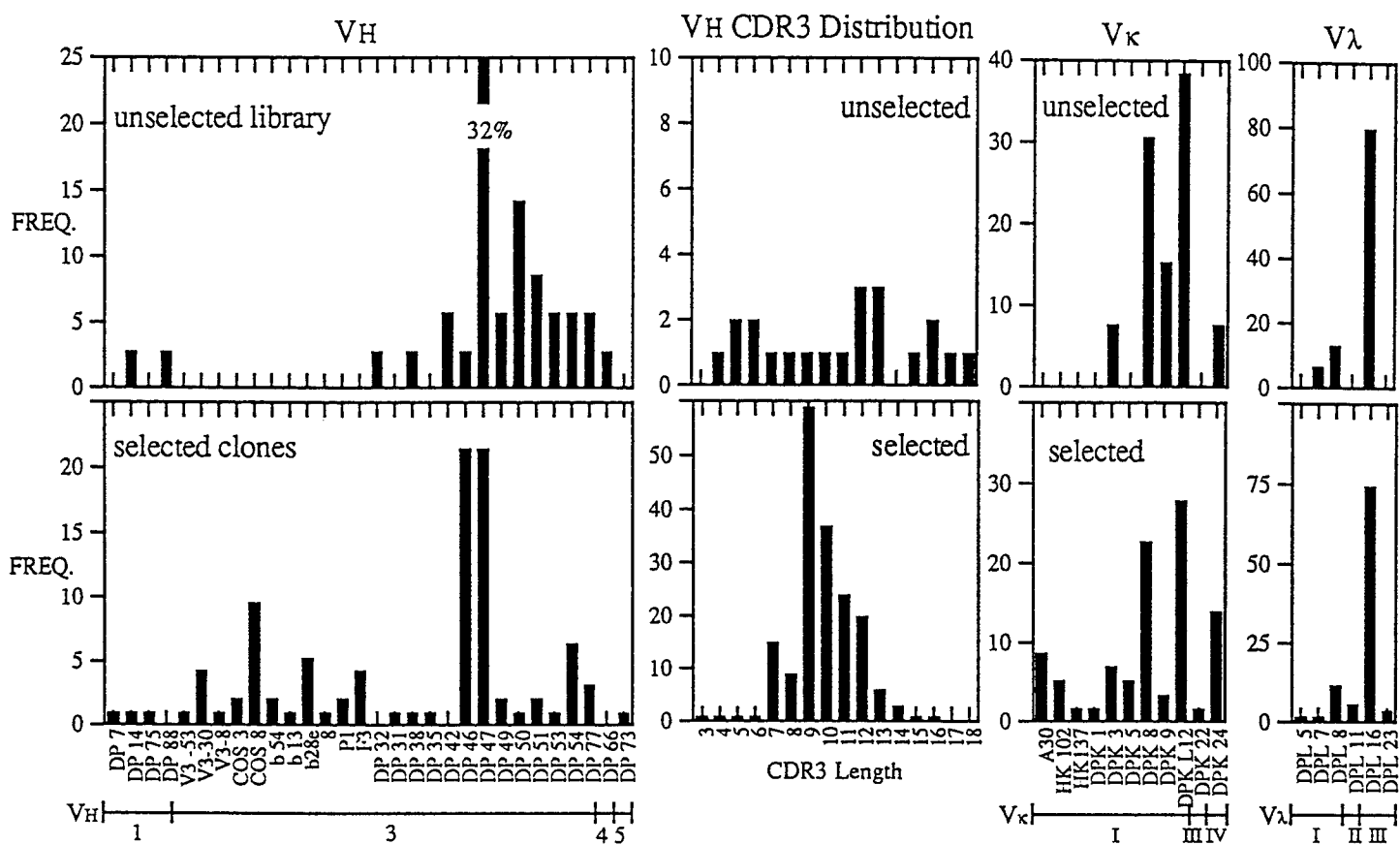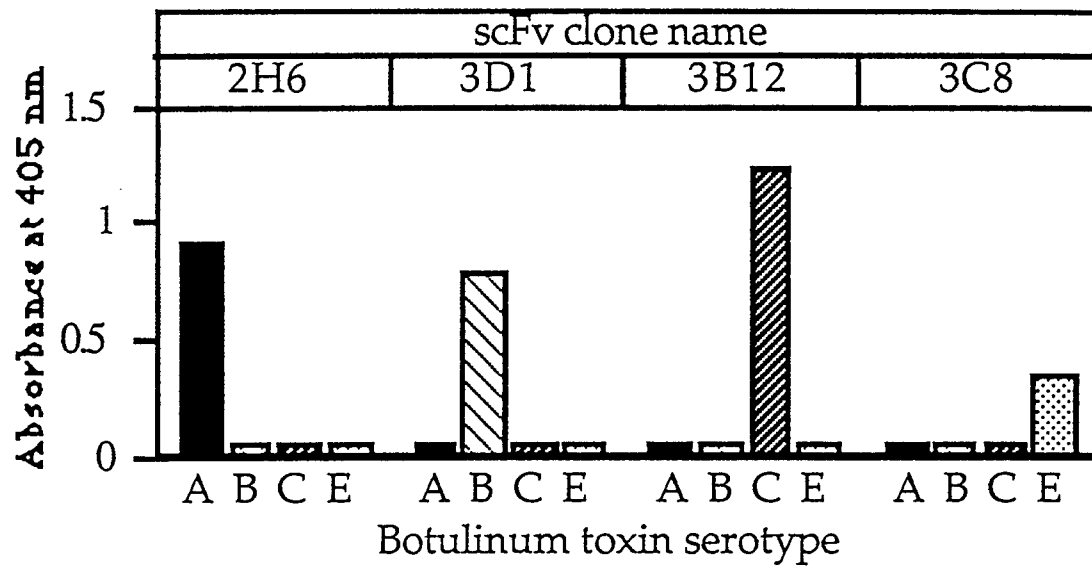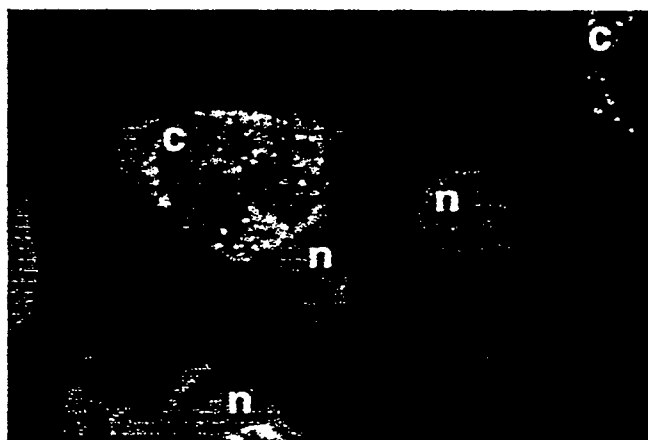
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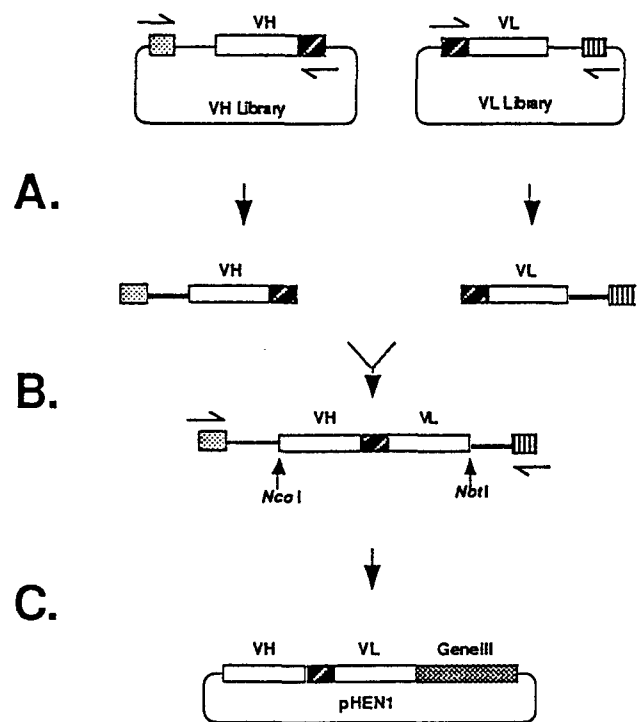
Figure 2

Figure 3.

Table 1.

Protein antigen used for selection	Percentage (number) of ELISA positive clones	Number of different antibodies isolated
FGF Receptor ECD	69 (18/26)	15
BMP Receptor Type I ECD	50 (12/24)	12
Activin Receptor Type I ECD	66 (16/24)	7
Activin Receptor Type II ECD	66 (16/24)	4
Erb-B2 ECD	91 (31/34)	14
VEGF	50 (48/96)	6
BoNT/A	28 (26/92)	14
BoNT-A C-fragment	95 (87/92)	10
BoNT/B	10 (9/92)	5
BoNT/C	12 (11/92)	5
BoNT/E	9 (8/92)	3
Bungarotoxin	67 (64/96)	15
Cytochrome b5	55 (53/96)	5
Chlamydia trachomatis EB	66 (63/96)	7

Table 2.

Specificity and clone	K_d (x 10^{-9}M)	k_{on} (x 10^5M^{-1}s^{-1})	k_{off} (x 10^{-3}s^{-1})
ErbB-2 B7A	0.22	4.42	0.1
ErbB-2 G11D	0.48	2.19	0.11
ErbB-2 A11A	0.49	3.69	0.18
ErbB-2 F5A	4.03	1.62	0.65
BoNT-A 2A9	26.1	0.25	0.66
BoNT-A 2H6	38.6	2.2	8.5
BoNT-A 3F6	66.0	4.7	30.9
BoNT-A 2B6	71.5	1.1	7.8

Table 3.

Library	Library size and type*	Number of protein antigens studied	Average number of antibodies per protein antigen	Number of affinities measured	Range of affinities for protein antigens K_d (x 10^{-9}M)
Marks et al (12)	3.0×10^7 (scFv, N)	2	2.5	1	100-2000
Nissim et al (13)	1.0×10^8 (scFv, SS)	15	2.6	ND	ND
deKruif et al (42)	3.6×10^8 (scFv, SS)	12	1.9	3	100 - 2500
Griffiths et al (14)	6.5×10^{10} (Fab, SS)	30	4.8	3	7 - 58
Vaughan et al (15)	1.4×10^{10} (scFv, N)	3	7.0	3	4.2 - 8.0
Sheets et al (this work)	6.7×10^9 (scFv, N)	14	8.7	8	0.22 - 71.5

Figure and Table Legends

Figure 1. Schematic outline of the approach used for library construction. A new library of V_H and genes was generated from rearranged human V-genes and cloned into the plasmid pCITE3A. The V_L genes used for scFv assembly were derived from a previously constructed scFv library contained in the plasmid plasmid pHEN1 (12). The vector containing the V_L repertoire also contained the scFv linker DNA 5' to the V_L genes. Primers for reamplification of the V-gene repertoires were derived from sequences several hundred base pairs 5' (the V_H genes) or 3' (the V_L genes) of the scFv gene cloning sites. This facilitated the efficiency of PCR assembling a new scFv repertoire and increasing the efficiency of cutting assembled scFv genes with restriction enzymes. **A.** V_H and linker-V_L gene repertoires were generated by PCR from the plasmid DNA of the separate libraries. The V_H genes were amplified using a plasmid specific primer (▭→) and an equimolar mixture of HuJH primers (←▨). The linker DNA and V_L genes were amplified using a plasmid specific primer (←▥) and an equimolar mixture of RHuJH primers (▨→). The RHuJH primers are complementary to the HuJH primers. **B.** The V_H and linker DNA-V_L gene repertoires were PCR assembled into a single chain Fv (scFv) gene repertoire. **C.** The assembled scFv gene repertoire was cut with the restriction enzymes *NcoI* and *NotI* and cloned into the plasmid pHEN1 (17) for phage-display.

Figure 2. V-gene usage and V_H CDR3 length of unselected and antigen specific scFv. The V_H and V_L genes were sequenced and the germline gene assigned based on homology to a database (VBASE) of germline V-genes compiled by Tomlinson et al (25). Specific V_H, V_κ and V_λ genes are listed on the ordinate, with the V_H, V_κ or V_λ germline gene family indicated below. Only V-genes in unselected or selected clones are listed.

Figure 3. Specificity of anti-Botulinum neurotoxin scFv. Representative scFv (2H6, 3D1, 3B12 and 3C8) isolated respectively from selections on Botulinum neurotoxin (BoNT) serotypes A, B, C and E were studied. Specificity was determined by ELISA.

Figure 4. Staining of HeLa cells infected with *Chlamydia trachomatis* with the scFv 2A10. The scFv specifically stains *Chlamydia trachomatis* elementary bodies (c) within infected HeLa cells but does not stain uninfected cells. n = nucleus.

Table 1. **Results of phage antibody library selections.** For each antigen (column 1), the number and the percentage of positive clones selected (column 2) and the number of different antibodies isolated (column 3) is indicated.

Table 2. **Affinities and binding kinetics of anti-BoNT A C-fragment and anti-Erb-B2 scFv.** Association (k_{on}) and dissociation (k_{off}) rate constants for purified scFv were measured using surface plasmon resonance (BIAcore) and K_d calculated as (k_{off}/k_{on}).

Table 3. Comparison of protein binding antibodies selected from non-immune phage-display antibody libraries. *
For library type, N = V-gene repertoires obtained from V-genes rearranged in vivo; SS = semi-synthetic V-genes constructed from cloned V-gene segments and synthetic oligonucleotides encoding V_H CDR3. ND = not determined.

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

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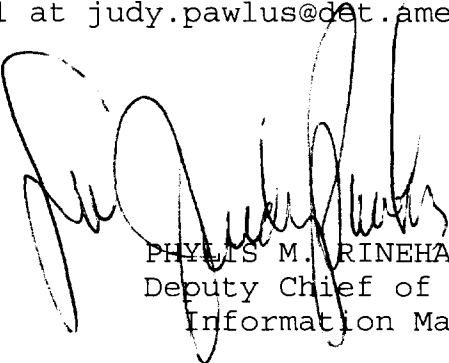VA 22060-6218

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