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13. ABSTRACT *(Maximum 200 Words)*

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, antibody phage display, to produce a new generation of tumor specific antibodies. We 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 previously achievable. Finally, we have developed methodologies which permit direct selection of libraries on tumor cells for the purpose of generating antibodies with desirable functional properties, such as endocytosis and growth inhibition. A panel of antibodies have been isolated which are breast tumor cell specific and a method has been developed where the antibody can be used to identify the receptor bound.

We have applied these technologies to produce several human antibodies that bind 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 tumors in mice. The National Cancer Institute Decision Network is supporting further pre-clinical development for anticipated clinical trials for breast cancer.

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1. Introduction

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

Despite the demonstration of antigens which are overexpressed on tumor cells, antibodies have been used with limited success for diagnosis and treatment of solid tumors, (reviewed in ref. (5, 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 $1.0x\ 10^{-9}$ 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.

2. Body of report

The work in the fourth year of the grant builds directly on results produced during the first three years 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, 2 and 3 reports. To facilitate distinction, each section is broken down by year, though the distinction is somewhat arbitrary.

Work during the first two years of the grant focused on selection of a 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 has been recently published (25).

In the first two years, work was also begun to identify the optimal means of increasing antibody fragment affinity (technical objectives 5 and 6) 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 has been recently published (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).

The major focus of work in the third and fourth 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). As a result, we have been able to develop a method which makes it possible to directly select internalizing antibodies which bind the selecting cell type but not the depleting cell type. This is the first example of successful selection of a non-immune phage antibody library directly on cells. The first two antibodies we have characterized in detail bind ErbB2 and the transferrin receptor. Unlike C6.5, this new anti-ErbB2 scFv (F5) is rapidly internalized by ErbB2 expressing cells and is more easily purified and conjugated to liposomes than C6.5. It also appears to have a greater therapeutic effect in preclinical models than C6.5. As a result, it is likely that F5 will be the targeting antibody for stealth liposomes developed in collaboration with the NCI for a phase I clinical trial rather than C6.5. Using the anti-transferrin scFv, we were able to develop a method to identify the antigen bound by the scFv. In fact, this is how we determined that this particular scFv bound the transferrin receptor. We are currently in the process of characterizing the many additional antibodies which appear to be tumor cell specific with respect to: 1) cell reactivity; 2) rate of internalization; 3) inhibitory effect on cell growth; and 4) antigen bound.

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

Year 1

A 3.0 x 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_κ, C_λ, and IgM primers, and 1st strand cDNA synthesized. 1st strand cDNA was used as a template for PCR amplification of the V_H, V_κ, and V_λ gene repertoires. The V_H gene repertoires were cloned into the vector pUC119Sfi-Not as NcoI-NotI fragments, to create a library of 8.0 x 10^8 members. The library was diverse by PCR fingerprinting. Single chain linker DNA was spliced onto the V_κ, and V_λ gene repertoires using PCR and the repertoire cloned as an XhoI-NotI fragment into the vector pHENIXscFv to create a library of 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

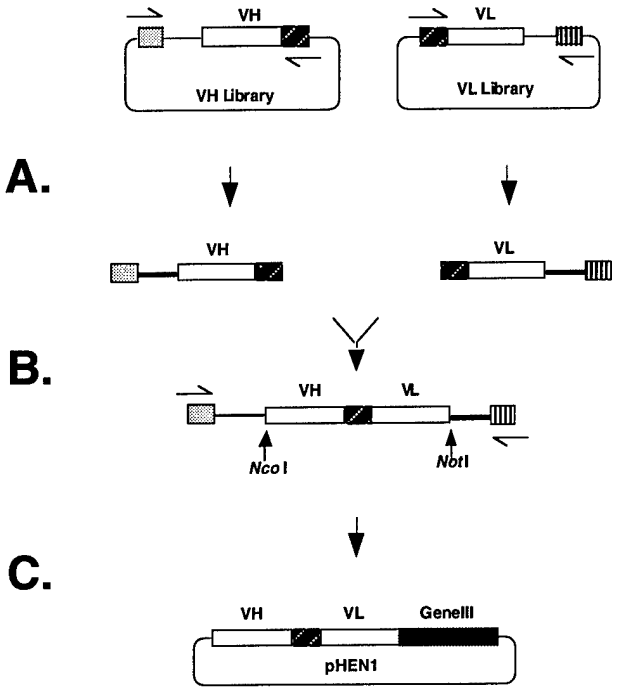

Figure 1. Method for construction of a large human scFv phage antibody library. The strategy for library construction involved optimizing the individual steps of library construction to increase both the efficiency of scFv gene assembly and to increase the efficiency of cloning assembled scFv genes. **(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 *Nco*I and *Not*I 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 $10^5 M^{-1}s^{-1}$)	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

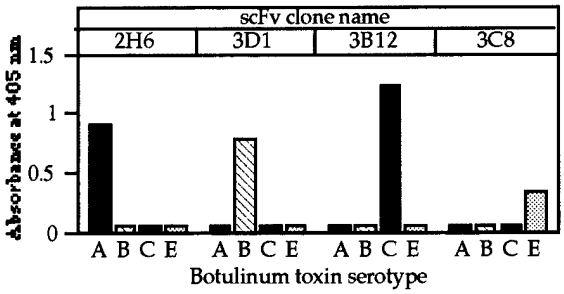

Figure 2. Specificity of anti-Botulinum (BoNT) scFv. scFv were isolated by selection of a phage antibody library on either BoNT serotype A (2H6), serotype B (3D1), serotype C (3B12) or serotype E (3C8). Specificity was determined by ELISA on the different serotypes. Each scFv was specific for the serotype used for selection.

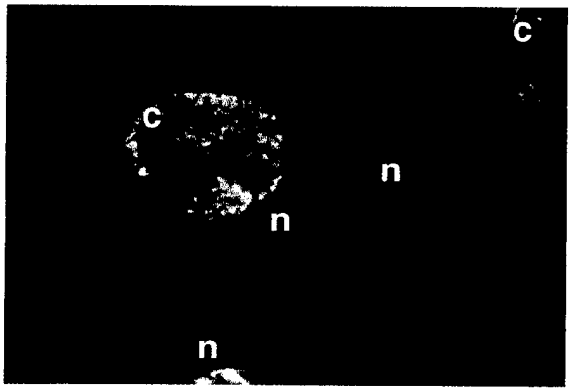

Figure 3. Specificity of anti-Chlamydial scFv. An anti-chlamydial scFv was isolated by direct selection of a phage antibody library on Chlamydial elementary bodies (EB). Purified scFv was used to stain chlamydial infected HeLa cells. c= chlamydial EB FITC stained. n = nucleus of HeLa cell.

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

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.

Significance: We have generated a high complexity 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.

Years 3 and 4

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).

Such a selection methodology relies on normal growth factor receptor biology; growth factor binding causes receptor activation via homo- or heterodimerization, either directly for bivalent ligand or by causing a conformational change in the receptor for monovalent ligand, and receptor mediated endocytosis (40). Antibodies can mimic this process, stimulate endocytosis, become internalized and be taken up into the cytosol. In general, this requires a bivalent antibody capable of mediating receptor dimerization (41, 42). In addition, the efficiency with which antibodies mediate internalization differs significantly depending on the epitope recognized (42, 43). Unlike multivalent IgG antibodies, phage antibody libraries typically display monomeric single chain Fv (scFv) or Fab antibody fragments fused to pIII as single copies on the phage surface using a phagemid system (16, 20, 25). Such monovalent display may be unlikely to lead to efficient receptor crosslinking and phage internalization. To determine the feasibility of selecting internalizing antibodies and to identify the most efficient display format, we studied the human scFv (C6.5) which binds ErbB2 (26). Using wild type C6.5 scFv, we demonstrate that anti-ErbB2 phage antibodies can undergo receptor mediated endocytosis. Using affinity mutants and dimeric diabodies of C6.5 displayed as either single or multiple copies on the phage surface, we defined the role of affinity, valency, and display format on phage endocytosis and identified the factors that lead to the greatest enrichment for internalization. The results indicate that it is possible to select for endocytosable antibodies, even at the low concentrations that would exist for a single phage antibody member in a library of 10^9 members.

2.3A. The model system utilized to study phage antibody internalization

C6.5 scFv binds ErbB2 with a $K_d = 1.6 \times 10^{-8}$ M and is a stable monomeric scFv in solution with no tendency to spontaneously dimerize or aggregate (26). To determine the impact of affinity on internalization, we studied a scFv (C6ML3-9) which differs from C6.5 by 3 amino acids (30). C6ML3-9 scFv is also a stable monomer in solution and binds the same epitope as C6.5 scFv but with a 16 fold lower K_d (1.0×10^{-9} M) (26, 30). Since receptor homodimerization appears to typically be requisite for antibody internalization we also studied the dimeric C6.5 diabody (32). Diabodies are scFv dimers where each chain consists of heavy (V_H) and light (V_L) chain variable domains connected using a peptide linker which is too short to permit pairing between domains on the same chain. Consequently, pairing occurs between complementary domains of two different chains, creating a stable noncovalent dimer with two binding sites (44). The C6.5 diabody was constructed by shortening the peptide linker between the Ig V_H and V_L domains from 15 to 5 amino acids and binds ErbB2 on SKBR3 cells bivalently with a K_d approximately 40 fold lower than C6.5 (4.0×10^{-10} M) (32).

Native C6.5 scFv and C6.5 diabody was expressed and purified from *E. coli* and analyzed for endocytosis into ErbB2 expressing SKBR3 breast tumor cells by immunofluorescent confocal microscopy. As expected, monomeric C6.5 scFv is not significantly internalized whereas the dimeric C6.5 diabody can be detected in the cytoplasm of all cells visualized (figure 4).

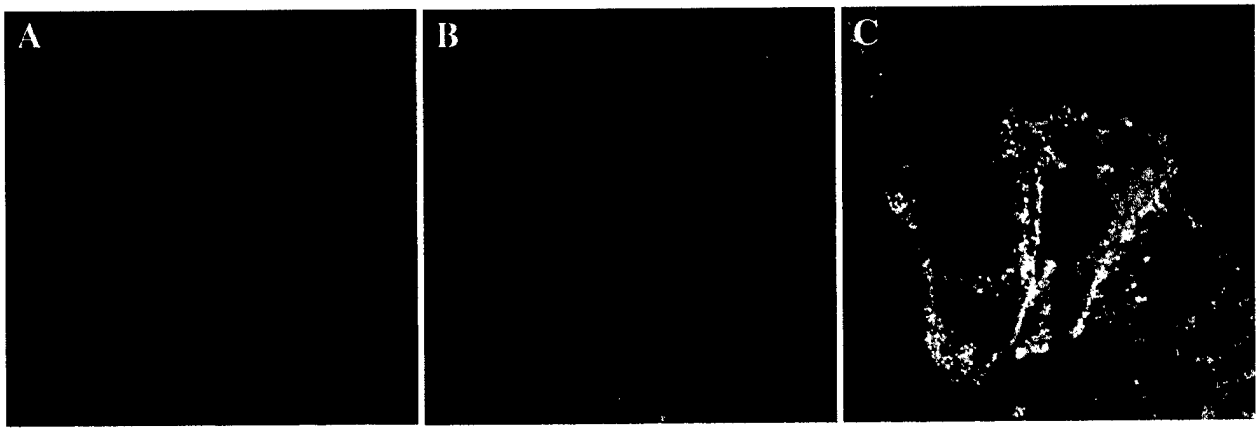

Figure 4. Internalization of soluble C6.5 scFv and diabody. SKBR3 cells grown on cover slips were incubated with (A) anti-botulinum scFv (B) C6.5 scFv or (C) C6.5 diabody for 2 hours at 37°C. Coverslips were washed with PBS and stripping buffer, cells were fixed and permeabilized and intracellular antibodies were detected by confocal microscopy using the anti-myc antibody 9E10, anti-mouse biotinylated antibody and streptavidin Texas-Red.

For subsequent experiments, the C6.5 and C6ML3-9 scFv and C6.5 diabody genes were subcloned for expression as pIII fusions in the phagemid pHEN-1 (15). This should yield phagemid predominantly expressing a single scFv or diabody-pIII fusion after rescue with helper phage (16) (figure 5A and B). Diabody phagemid display a bivalent antibody fragment resulting from intermolecular pairing of one scFv-pIII fusion molecule and one native scFv molecule (figure 5B). The C6.5 scFv gene was also subcloned into the phage vector fd-Sfi/Not. This results in phage with 3 to 5 copies each of scFv-pIII fusion protein (figure 5C). The human breast cancer cell line SKBR3 was used as a target cell line for endocytosis. Its surface ErbB2 density is approximately 1.0×10^6 per cell.

2.3B. C6.5 phagemids are endocytosed by human cells expressing ErbB2

C6.5 scFv phagemids were incubated for 2 hours with SKBR3 cells grown on coverslips at 37°C to allow active internalization. Cells were extensively washed with PBS to remove non specific binding and washed an additional three times with high salt and low pH (stripping) buffer to remove phage specifically bound to cell surface receptors. Internalized phagemid

were detected with a biotinylated M13 antiserum recognizing the major coat phage protein pVIII. An anti-botulinum toxin phagemid was used as a negative control. Staining was analyzed by using immunofluorescent microscopy (Figure 6). Approximately 1% of the cells incubated with C6.5 scFv phagemid showed a strong intracellular staining consistent with endosomal localization (figure 6B) while no staining was observed for anti-botulinum phagemid (figure 6A). Furthermore, no staining was seen if the incubation was performed for 2 hours at 4°C instead of 37°C (data not shown). Staining performed after the PBS washes but before washing with stripping buffer showed membrane staining of all the cells, indicating that multiple washes with stripping buffer is necessary to remove surface bound phagemids. The results also indicate that only a fraction of the cell bound phage are endocytosed.

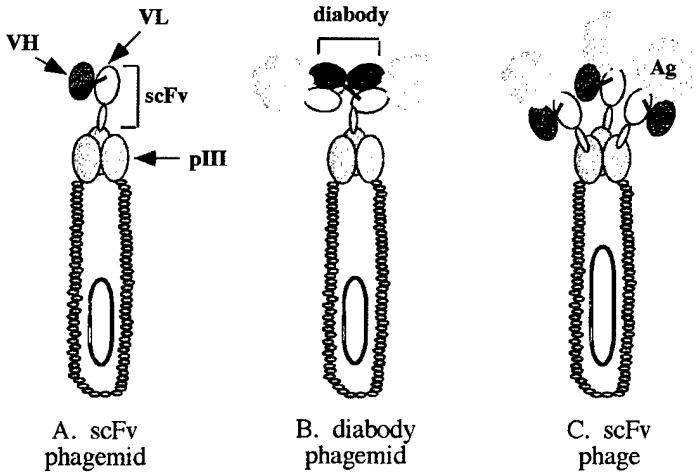

Figure 5. Antibody phage display: Cartoon of phage displaying (A) a single scFv (B) a single diabody or (C) multiple scFv. scFv = single chain Fv antibody fragment; V_H = Ig heavy chain variable domain; V_L = Ig light chain variable domain; pIII = phage minor coat protein pIII; Ag = antigen bound by scFv.

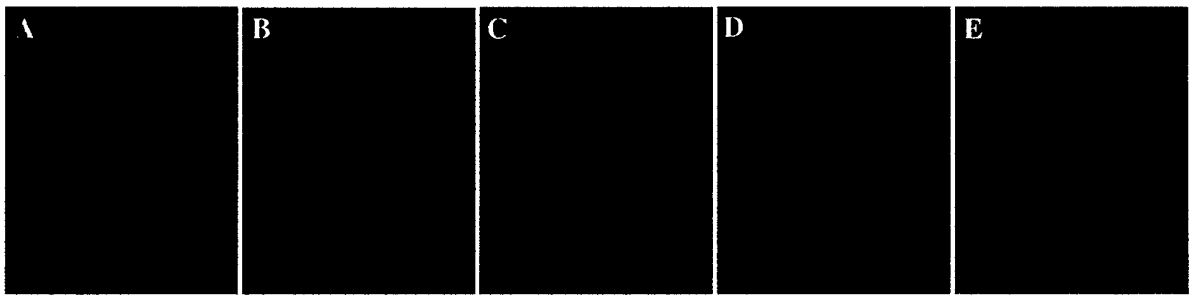

Figure 6. Internalization of C6.5 phage derivatives. SKBR3 cells grown on coverslips were incubated with 5.0 x 10^{11} cfu/ml (A) anti-botulinum phagemid (B) scFv C6.5 phagemid (C) C6ML3-9 phagemid (D) C6.5 diabody phagemid or (E) C6.5 phage for 2 hours at 37°C. The cells were treated as described in figure 4 and intracellular phage were detected with a fluorescent microscope using biotinylated anti-M13 antiserum and Texas-Red streptavidin.

2.3C. Increased affinity and bivalency lead to increased phage endocytosis

We compared the internalization of C6.5 scFv, C6ML3-9 scFv and C6.5 diabody phagemid and C6.5 scFv phage using immunofluorescence. Both C6ML3-9 scFv and C6.5 diabody phagemid as well as C6.5 scFv phage yielded increased intensity of immunofluorescence observed at the cell surface compared to C6.5 scFv phagemid. For C6ML3-9 scFv phagemid, approximately 10% of the cells showed intracellular fluorescence after 2 hours of incubation (Figure 6C). This value increased to approximately 30% of cells for the dimeric C6.5 diabody phagemid (figure 3D) and 100% of cells for multivalent C6.5 scFv phage (figure 6E).

2.3D. Infectious phage can be recovered from within the cell and their titer correlates with the level of uptake observed using immunofluorescence

To determine if infectious phage antibody particles could be recovered from within the cell, we incubated approximately 5.0×10^5 SKBR-3 cells for 2 hours at 37°C with 3.0×10^{11} cfu of the different phagemid or phage. Six PBS washes were used to remove non-specifically bound phage and specifically bound phage were removed from the cell surface by three consecutive washes with stripping buffer (washes I, II and III respectively, Table 4) . The cells were then lysed with 1 mL of a 100 mM triethylamine solution (TEA) (representing the intracellular phage). The three stripping washes and the cell lysate were neutralized and their phage titer was determined by infection of *E. coli* TG1. The titers of phage recovery are reported in Table 4.

Table 4. Titration of membrane bound and intracellular phage. 3.0×10^{11} cfu of monovalent C6.5 scFv phagemid, 16 fold higher affinity monovalent C6ML3-9 scFv phagemid, bivalent C6.5 diabody phagemid or multivalent C6.5 fd phage were incubated with SKBR3 cells for 2 hours at 37°C. Cells were washed 6 times with PBS, 3 times with stripping buffer and then lysed to recover intracellular phage. The various fractions were neutralized and the phage titered. The total number of cfu of each fraction is reported. Non specific anti-botulinum phagemid were used to determine non specific recovery.

Phage Antibody	*Cell Surface Phage Titer ($\times 10^{-5}$)*			*Intracellular*
	1st Wash	*2nd Wash*	*3rd Wash*	*Phage Titer ($\times 10^{-5}$)*
Anti-botulinum phagemid	280	36	2.8	15
C6.5 scFv phagemid	600	96	7.6	52
C6ML3-9 scFv phagemid	2500	140	32	270
C6.5 diabody phagemid	1800	120	13	450
C6.5 scFv phage	2300	620	56	2200

Considerable background binding was observed in the first stripping wash for the anti-botulinum phage even after 6 PBS washes (2.8×10^7 cfu, Table 4). This value likely represents phage non-specifically bound to the cell surface as well as phage trapped in the extracellular matrix. The amount of surface bound phage increased only 2.1 fold above this background for C6.5 scFv phagemid (Tables 4 and 5). With increasing affinity and avidity of the displayed C6.5 antibody fragment, the titer of cell surface bound phagemid or phage increased (Table 4). The titer of phage in the consecutive stripping washes decreased approximately 10 fold with each wash. These additional stripping washes led to a minor increase in the titer of specific phage eluted compared to the background binding of the anti-botulinum phage (2.7 fold for C6.5 scFv phagemid to 20 fold for C6.5 scFv phage, Table 5). The only exception was the titer of the C6.5 diabody phagemid, where the ratio actually decreased from 6.4 fold to 4.6 fold. This is likely due to the fact that in the diabody the V_H and V_L domains that comprise a single binding site are not covalently attached to each other via the peptide linker. This increases the likelihood that a stringent eluent (like glycine) could dissociate V_H from V_L and abrogate binding to antigen.

Three stripping washes were required to ensure that the titer of phage recovered after cell lysis was greater than the titer in the last stripping wash (Table 4). We presumed that after three stripping washes, the majority of the phage eluted represented infectious particles from within the cell rather than from the cell surface. In fact, since the cell lysate titer observed with non-specific anti-botulinum phage was considerable (1.5×10^6) and greater than observed in the last stripping wash, it is likely that many phage remain trapped within the extracellular matrix and relatively inaccessible to the stripping buffer washes. Some anti-botulinum phage might also be non-specifically endocytosed by cells, but this is likely to be a small amount given the immunofluorescence results (figure 6). The titer of phage in the TEA fraction increased with increasing affinity and avidity of C6.5, with the highest titers observed for the dimeric C6.5

diabody phagemid and the multivalent C6.5 scFv phage (Table 4). The values represent a 30 fold (C6.5 diabody phagemid) and 146 fold (C6.5 scFv phage) increase in titer compared to the anti-botulinum phage (Table 4). We have presumed that the increase in the phage titer in the cell lysate compared to the last stripping wash is due to endocytosed phage. In fact, some of these phage could have come from the cell surface or intracellular matrix. While this could be true for a fraction of the phage from the cell lysate, the immunofluorescence results indicate that at least some of the phage are endocytosed. One indicator of the relative fraction of endocytosed phage for the different C6.5 molecules is to compare the amount of phage remaining on the cell surface prior to cell lysis (last stripping wash) with the amount recovered after cell lysis. This ratio shows only a minor increase for monovalent C6.5 scFv or C6ML3-9 scFv phagemid (6.8 and 8.4 fold respectively) compared to anti-botulinum phagemid (5.4) (Table 5). In contrast the ratios for dimeric C6.5 diabody phagemid and multivalent C6.5 scFv phage increase to a greater extent (35 and 39 respectively) compared to anti-botulinum phagemid.

Table 5. Specific enrichment of anti-ErbB2 phage compared to anti-botulinum phage. *The titers of anti-ErbB2 phage are divided by the titers of the anti-botulinum phage (Table 4) to derive an enrichment ratio for specific vs nonspecific binding or internalization. **The titer of intracellular phage is divided by the titer of cell surface bound phage (Table 4) to derive the ratio of internalized phage vs surface bound phage.

Phage Antibody	*Anti-ErbB2 /Anti-Botulinum Phage Titer Ratio**			*Intracellular/ Cell Surface Phage Ratio***
	Cell surface (1st Wash)	*Cell surface (3rd Wash)*	*Intracellular*	
C6.5 scFv phagemid	2.14	2.7	3.5	6.8
C6ML3-9 scFv phagemid	8.9	11.4	18	8.4
C6.5 diabody phagemid	6.4	4.6	30	35
C6.5 scFv phage	8.2	20	146	39

2.3E. Reducing the background of non-internalized phage

To reduce the background of non-specific phage recovery, we studied the effect of trypsinizing the cells prior to TEA lysis. This should remove phage trapped in the extracellular matrix. Trypsinization also dissociates the cells from the cell culture flask, permitting transfer to a new vessel and elimination of any phage bound to the cell culture flask. For these experiments, C6.5 scFv phagemid (5.0×10^8 ampicillin resistant cfu) were mixed with a 1000 fold excess of wild type fd phage (5.0×10^{11} tetracylcine resistant cfu). After incubation of phagemid with SKBR-3 cells for 2 hours at 37°C, cells were washed with PBS and three times with stripping buffer. Cells were then directly lysed with TEA or treated with trypsin, washed twice with PBS and then lysed with TEA. Phagemid in the first stripping wash and the cell lysate were titered by infection of *E. coli* TG1 and plated on ampicillin and tetracycline plates. The titer of fd phage and C6.5 scFv phagemid recovered from the cell surface was comparable for the two experimental groups (Figure 7). The ratio of fd phage/C6.5 scFv phagemid in the cell surface fractions (160/1 and 250/1) yields a 4 to 6 fold enrichment achieved by specific cell surface binding from the initial 1000 fold ratio. Without trypsinization, the ratio of fd phage/C6.5 scFv phagemid in the cell lysate increases only 6.1 fold; in contrast, the ratio increases 209 fold with trypsinization (figure 7). This results from a 60 fold reduction in non-specific binding with only a minor reduction in the amount of specific phage recovery (figure 7).

2.3F. Recovery of internalized phage at low phage concentrations

Only very large phage antibody libraries containing more than 5.0×10^9 members are capable of generating panels of high affinity antibodies to all antigens (25, 38). Since phage can only be concentrated to approximately 10^{13} cfu/ml, a typical phage preparation from a large library will only contain 10^4 copies of each member. Thus selection of libraries for endocytosis

could only work if phage can be recovered when applied to cells at titers as low as 10^4. We therefore determined the recovery of infectious phage from within SKBR3 cells as a function of the phage titer applied. SKBR3 cells were incubated with C6.5 scFv, C6ML3-9 scFv or C6.5 diabody phagemids or C6.5 scFv phage for 2 hours at 37°C. Cells were washed three times with stripping buffer, trypsinized and washed twice with PBS. Cells were lysed and intracellular phage titered on *E. coli* TG1. Phage recovery increased with increasing phage titer for all phage studied (figure 8). For monovalently displayed antibodies, phagemid could not be recovered from within the cell at input titers less than 3.0 x 10^5 (C6.5 scFv) to 3.0 x 10^6 (C6ML3-9 scFv) This threshold decreased for bivalent and multivalent display (3.0 x 10^4 for C6.5 diabody phagemid and C6.5 scFv phage).

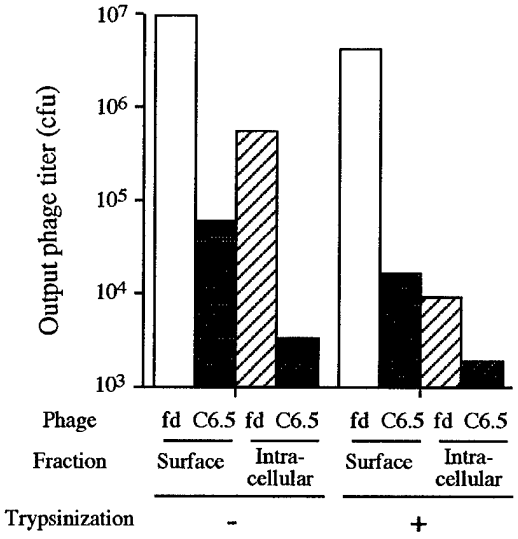

Figure 7. Effect of trypsinization on the enrichment of antigen specific phage. A mixture of fd phage (5.0 x 10^{11} cfu) and C6.5 scFv phagemid (5.0 x 10^8 fu) was incubated with SKBR3 cells for 2 hours at 37°C. Washes were performed either as described above (-) or cells were trypsinized prior to cell lysis (+). Phage present in the first stripping buffer wash (cell surface phage) and the cell lysate (intracellular phage) were titered in the presence of ampicillin (C6.5 phagemid) or tetracycline (fd phage).

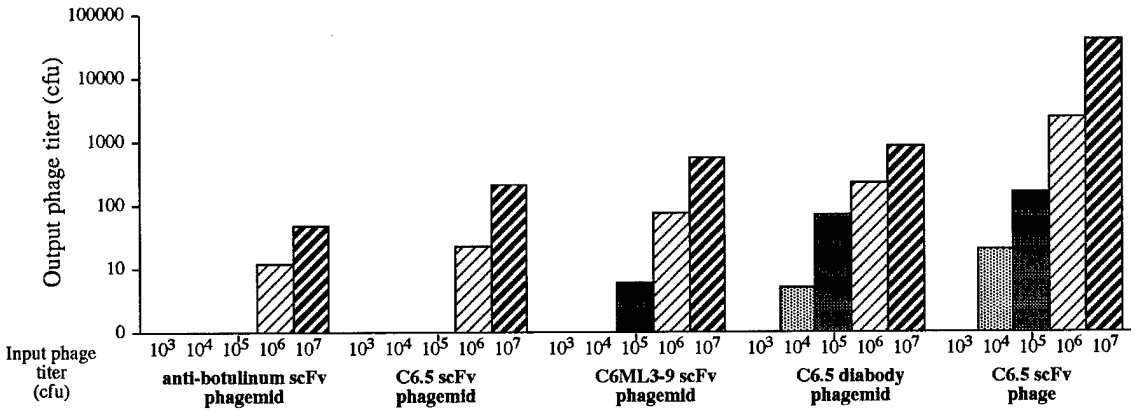

Figure 8. Effect of phage concentration on the recovery of intracellular phage. Various concentrations of C6.5 scFv phagemid, C6ML3-9 scFv phagemid, C6.5 diabody phagemid or C6.5 scFv phage (input phage titer) were incubated with subconfluent SKBR3 cells grown in 6-well plates for 2 hours at 37°C. Cells were treated as described above including the trypsinization step and intracellular phage were titered (output phage titer).

The results demonstrate for the first time that phage displaying an anti-receptor antibody can be specifically endocytosed by receptor expressing cells and can be recovered from the cytosol in infectious form. The results demonstrate the feasibility of directly selecting internalizing antibodies from large non-immune phage libraries and identify the factors that will lead to successful selections. When monovalent scFv antibody fragments were displayed

monovalently in a phagemid system, recovery of internalized phage was only 3.5 to 18 fold above background. Display of bivalent diabody or multivalent display of scFv in a phage vector increased recovery of internalized phage to 30 to 146 fold above background. This result is consistent with our studies of native monomeric C6.5 scFv and dimeric C6.5 diabody as well as studies of other monoclonal anti-ErbB2 antibodies where dimeric IgG but not monomeric Fab dimerize and activate the receptor and undergo endocytosis (42, 43). In fact it is likely that endocytosis of C6.5 and C6ML3-9 scFv phagemids reflect the small percentage of phage displaying two or more scFv (16). The importance of valency in mediating either high avidity binding or receptor crosslinking and subsequent endocytosis is confirmed by the only other report demonstrating specific phage endocytosis. Phage displaying approximately 300 copies of a high affinity Arg-Gly-Asp integrin binding peptide on pVIII were efficiently endocytosed by mammalian cells (39). Recovery of phage after endocytosis also increases the specificity of cell selections compared to recovery of phage from the cell surface. Thus enrichment ratios for specific vs non-specific surface binding range from 2 to 20 fold. These values are comparable to the approximately 10 fold enrichment reported by others for a single round of cell surface selection (45, 46). In contrast our enrichment ratios for specific vs non-specific endocytosis range from 3.5 to 146 fold.

Significance: Based on these results, selection of internalizing antibodies from phage antibody libraries would be most successful with either homodimeric diabodies in a phagemid vector or multivalent scFv using a phage vector Multivalent libraries would present the antibody fragment in the form most likely to crosslink receptor and undergo endocytosis. Antibodies from such libraries would need to be bivalent to mediate endocytosis. Alternatively, monomeric receptor ligands can activate receptors and undergo endocytosis, either by causing a conformational change in the receptor favoring the dimeric form or by simultaneously binding two receptors. Monomeric scFv that bound receptor in a similar manner could also be endocytosed. Thus selection of libraries of monovalent scFv in a phagemid vector could result in the selection of ligand mimetics that activate receptors and are endocytosed as monomers. Such scFv could be especially useful for the construction of fusion molecules for the delivery of drugs, toxins or DNA into the cytoplasm. Since antibodies which mediate receptor internalization can cause receptor down regulation and growth inhibition (43, 47-49), selection for endocytosable antibodies may also identify antibodies which directly inhibit or modulate cell growth.

2.3G. 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 grown adherent to subconfluency in a tissue culture flask. For selection, subconfluent SKBR3 cells were incubated with 3.0×10^{12} cfu of library phage in the presence of approximately 1 million human fibroblasts in suspension for 1.5 hours. This step was performed at 4°C to allow the phage to bind without internalization. The fibroblasts were used to deplete the library of binders common to SKBR3 cells and fibroblasts (non tumor specific antigens). Subsequently, the cells were washed extensively with PBS to remove unbound phage and then incubated at 37°C for 15 minutes to allow endocytosis of surface bound phage antibodies. To recover intracellular phage, cells were: 1) stripped of surface bound phage using low pH glycine buffer; 2) trypsinized and transferred to a new vessel and washed again with PBS to remove phage trapped in the extracellular matrix; and 3) lysed to release internalized phage. The lysate was used to infect *E. coli* TG1 to prepare phage for the next round of selection. Three rounds of selection were performed. After each round of selection, the titer of intracellular phage increased along with the number of phage recovered per cell (Table 6). This suggests that cell selection was occurring. To analyze the results of the selection, ninety six 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. Clones positive for ErbB2 binding and SKBR3 binding were identified after the second round (Table 6). After the third round, 41% of the clones were positive for SKBR3 cell binding and 17% bound ErbB2 (Table 6).

Table 6. 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	2.73×10^6	0.013	ND	ND
2	1.2×10^5	3.3×10^6	0.038	12%	5%
3	7.5×10^6	2.0×10^6	3.75	41%	21%

To estimate the number of unique binders, the scFv gene from all ErbB2 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 (F5 and C1). Interestingly, neither of these scFv had a sequence identical to the 14 scFv isolated by selecting the same library on recombinant ErbB2 ECD (Table 1). Specificity for ErbB2 was confirmed by ELISA on a panel of 10 proteins as well as on ErbB2 and non-ErbB2 expressing cell lines (Table 2 and figures 9 and 10).

All scFv positive by SKBR3 ELISA but which did not bind ErbB2 were analyzed for binding to human fibroblasts by ELISA. None of these clones bound fibroblasts. A sample of 18 of these were analyzed by BstN1 fingerprinting, revealing 10 unique fingerprints. To examine the specificity of these scFv, phage were prepared and used to stain a panel of human cell lines (high ErbB2 expressing SKBR3 cells, moderate ErbB2 expressing SKOV3 cells (ovarian tumor cell line), low ErbB2 expressing MCF7 cells(breast tumor cell line), LNCaP cells (prostate tumor cell line), and fibroblasts) (Table 2 and figures 9 and 10).

Table 7. Fluorescent activated cell scanning (FACS) of individual phage antibodies to identify specificity.

Primary antibody	Cell type				
ErbB2 positive phage	*SKBR3*	*MCF7*	*SKOV3*	*LNCaP*	*Fibroblasts*
C6.5	526*	16	670	nd	1
F5	4867	123	5839	nd	11
C1	858	0	566	nd	0
4D5 (anti-ErbB2 control)	600	29	586	nd	2
ErbB2 negative phage					
3TE3	1056	1002	416	nd	130
H7	4003	1219	945	nd	93
3TB5	225	301	336	199	9
2TF5	1973	495	805	nd	0
3TH8	153	1	353	1	0
3TG5	469	80	714	82	3
3TF12	611	83	31	233	7
2TB4	138	3	1	nd	1
C2-1	181	8	81	nd	1
3GD8	103	6	1154	45	0

All phage stained SKBR3 cells with a signal intensity at least 40 times higher than fibroblasts (except for clone 3TE3). Several clones appear to be pan tumor specific (3TE3, H7, 3TB5 and 2TF5) while others are more selective in their binding pattern (3TH8 and 3TG5, SKBR3 and SKOV3 cells; 3TF12, SKBR3 and LNCaP cells; 2TB4 SKBR3 cells or 3GD8, SKOV3 cells).

In figure 9 (below) 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. In figure 10 (next page), 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 9, 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.

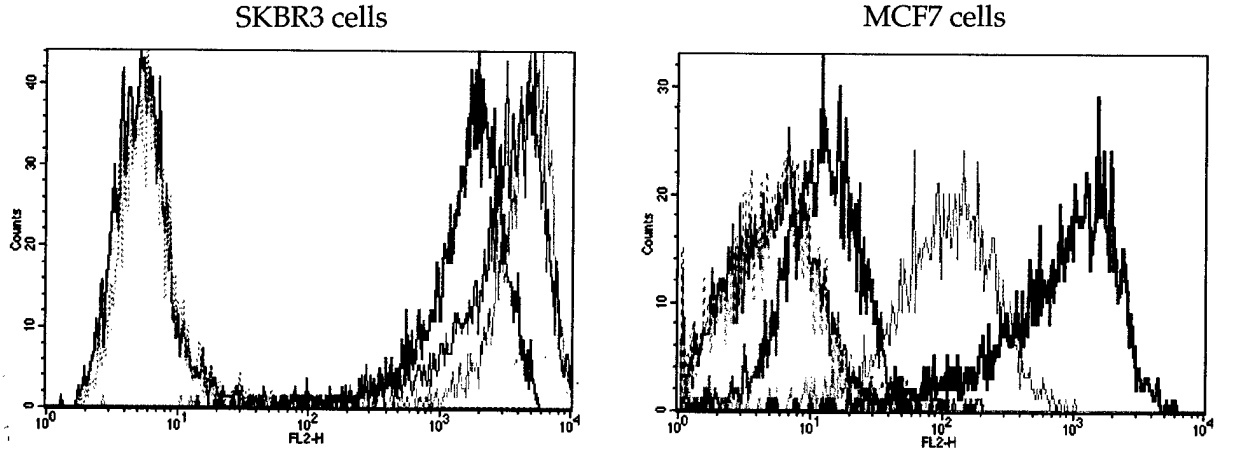

Figure 9. Staining of SKBR3 and MCF7 cells with phage antibodies as analyzed by FACS. 5.0 x 10^{12} phage/ml were incubated with cells. Binding was detected using biotinylated sheep anti-M13 and streptavidin-Texas red. Gray = 1° and 2° antibodies only, pink = anti-botulinum phage, green = C6.5-phage, orange = 3TF5 phage (anti-ErbB2), blue = 3GH7 phage.

2.3G1. Phage antibodies and native scFv are efficiently internalized

Since phage were selected on the basis of internalization, the selected phage should be rapidly endocytosed by SKBR3 cells. To examine this hypothesis, we studied two phage antibodies 3TF5 (F5, anti-ErbB2) and 3GH7 (H7, non ErbB2 binder). After a 2 hour incubation with F5 or G7 phage antibodies, all SKBR3 cells showed strong intracellular staining as determined by confocal microscopy (figure 11B and C). No staining was observed with an anti-botulinum phage antibody (figure 11C). As previously reported, only about 1% of the cells were stained by the anti-ErbB2 phage antibody C6.5, isolated by selection on recombinant ErbB2 ECD (figure 11D). To verify that native scFv had similar endocytosis properties, the internalization experiments were repeated using native F5 and H7 scFv purified as described above. After purification by immobilized metal affinity chromatography, gel filtration demonstrated that both scFv were monomeric with only small amounts of dimeric scFv observed. The monomeric fraction was collected and used immediately for internalization

studies. Both F5 and G7 scFv were taken up by more than 95% of SKBR3 cells (figure 12) compared to only 1% of cells taking up C6.5 scFv (figure 4).

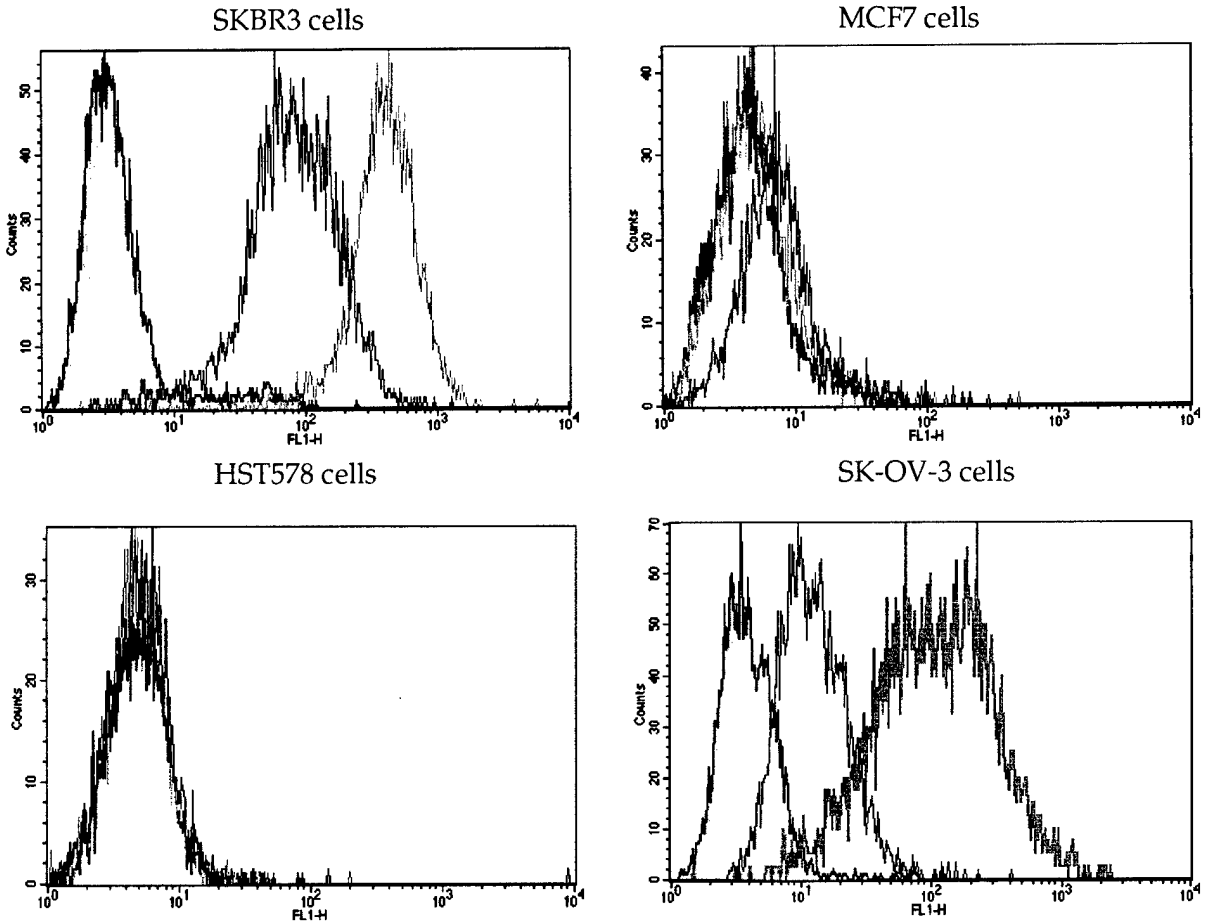

Figure 10. Staining of SKBR3, SK-OV-3, MCF7 and HST578 cells with scFv as analyzed by FACS. Cells were incubated with purified scFv (0.5 uM) and binding detected using mAb 9E10 (recognizes C-terminal myc epitope tag) and FITC-goat anti-mouse Fc. Gray = 1° and 2° antibodies only, orange = 3TF5 scFv (anti-ErbB2), blue = 3GH7 scFv.

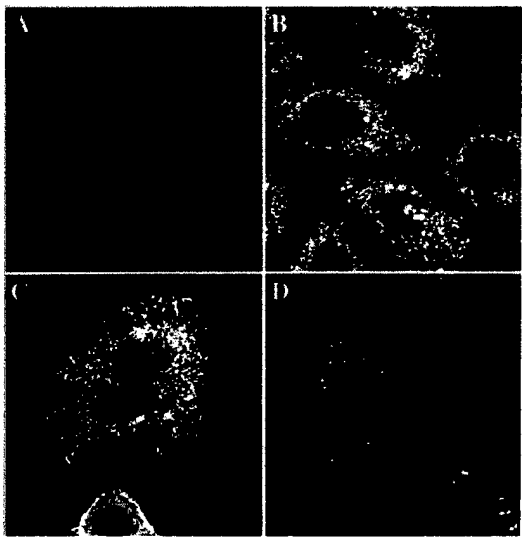

Figure 11. Internalization of phage as determined by immunofluorescence and confocal microscopy. SKBR3 cells were incubated with anti-botulinum phage (A), F5 phage (B), H7 phage (C) or C6.5 phage (D) at 37°C. Cells were washed with PBS followed by low pH glycine to remove surface bound phage antibody. Phage antibodies were visualized using confocal microscopy and biotinylated anti-M13 antibody and streptavidin Texas red.

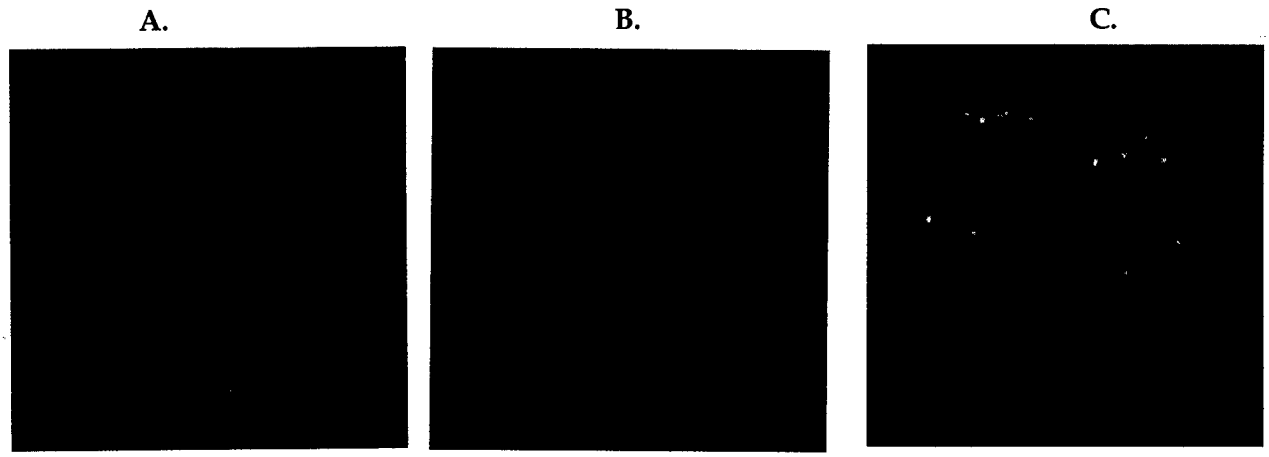

Figure 12. Internalization of native scFv by ErbB2 expressing SKBR3 cells. Cells were incubated with FITC labeled F5 scFv (A) and Texas Red labeled H7 scFv (B) for 2 hours. Cells were fixed, permeabilized and examined by Delta vision microscopy. Both scFv are internalized into greater than 95% of cells. When the images are superimposed for the two different filters (C) the scFv co-localize indicating a similar trafficking pathway.

2.3G2. Further characterization of ErbB2 binding scFv F5 and C1

Two unique phage antibodies were identified which were internalized by SKRB3 cells (F5 (described above) and C1 (referred to in section 2.3G). Neither of these phage were isolated when the same library was selected on recombinant ErbB2 (see section 2.2, Table 2). To determine why, the K_d of F5 (3.2×10^{-7} M) and C1 ($K_d = 1.0 \times 10^{-6}$ M) were measured. These K_d are significantly higher than the K_d measured for four of the scFv selected on recombinant ErbB2 (K_d = 0.1 to 0.65 nM). The higher K_d internalizing phage antibodies would have to compete with the lower K_d non-internalizing phage antibodies for selection on recombinant ErbB2 and were likely lost during the selection process. Since antibodies which are internalized as monomers are likely to be rare, and since there will be many more phage antibodies of lower affinity than higher affinity in a library, it is not surprising that the internalizing antibodies are of high K_d. Since antibodies which are internalized are likely to be rare, we hypothesized that it was likely that F5 and C1 recognized the same epitope. This was confirmed using a competition assay (figure 13). Thus as hypothesized, F5 and C1 recognize the same epitope, and a different epitope than C6.5.

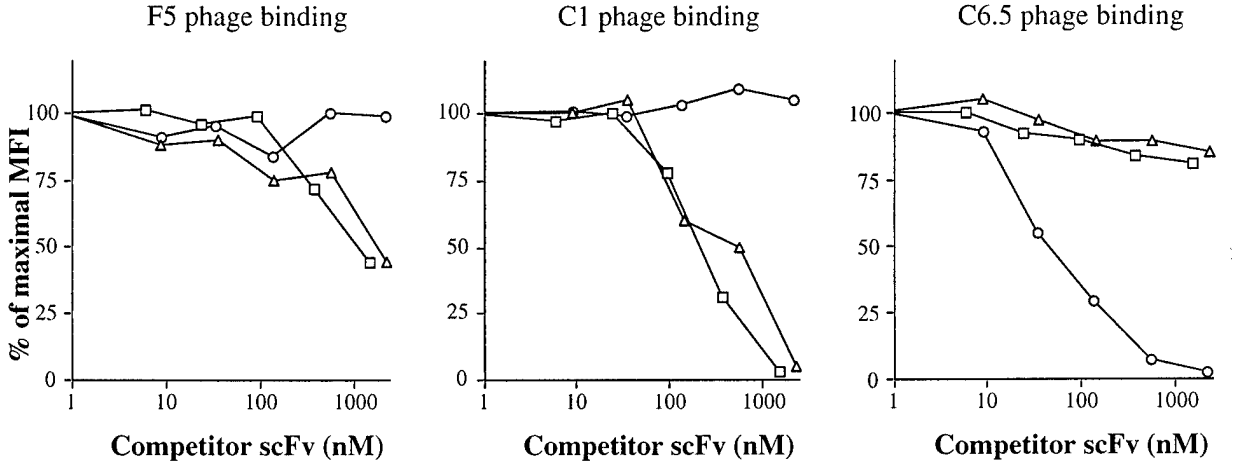

Figure 13. Epitope mapping of F5, C1 and C6.5 scFv. Inhibition of binding of F5 phage (left panel), C1 phage (center panel) or C6.5 phage (right panel) by increasing concentrations of soluble scFv-F5 (□), scFv-C1 (△) or scFv-C6.5 (○). Bound phages were detected with an anti-M13 biotinylated Ab and streptavidin-PE. Results are expressed in % of maximal mean fluorescent intensity (MFI).

As antibody induced internalization can potentially increase the turn over of ErbB2 receptors, reduce the density of cell surface receptors and have an effect on cell growth (50, 51), we tested the effects of scFv-F5 on SKBR3 cell growth. scFv-F5 had no effect on cell growth at a concentration of 300 nM (10 µg/ml) while the control mAb 4D5 inhibited cell growth of 50% after 5 days of culture at a concentration of 5 nM as previously published (50) (Figure 14). Since no inhibitory effect had been observed with monovalent derivatives of growth inhibitory ErbB2 antibodies (50, 52), we constructed a bivalent format scFv-F5 (with the scFv monomers linked by a C-terminal disulfide bond) whose size was checked by gel filtration. The dimeric F5 had no effect on SKBR3 growth (Figure 14). Similarly, neither scFv C6.5 or dimeric diabody C6.5 inhibited SKBR3 growth. We also tested the ability of F5 scFv to induce downstream signaling upon ErbB2 binding. Starved CHO-ErbB2 cells were stimulated with monovalent (scFv) and bivalent (diabody) formats of F5. Both induced weak phosphorylation of ErbB2 on tyrosine (data not shown). The bivalent format of F5 was able to slightly activate the MAP kinase cascade as shown by SDS-PAGE band shift using an anti-Erk antibody (data not shown).

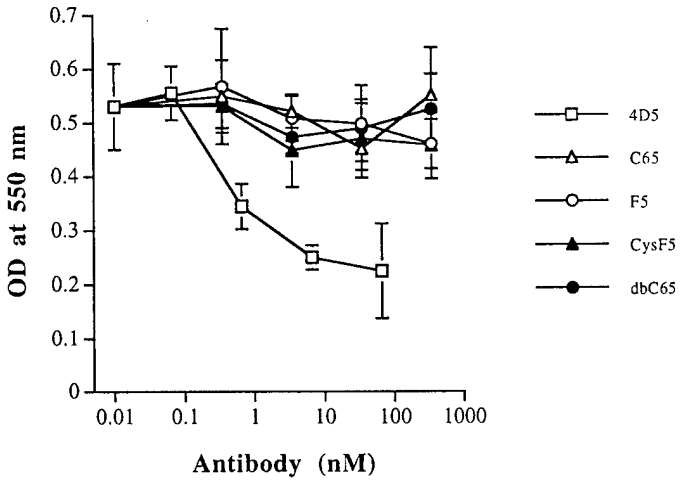

Figure 14. Effect of different antibodies on the growth of SKBR3 cells. Antibodies were added to cells in 96 well plates and incubated for 5 days. The number of living cells was estimated using the CellTiter growth assay kit (Promega) and reported as OD_{550}. 4D5 = Mab 4D5, C6.5 = C6.5 scFv, F5 = F5 scFv, Cys F5 = dimeric F5 scFvs, db C6.5 = C6.5 diabody.

2.3G3. Further characterization of non-ErbB2 binding scFv

For the 10 non-ErbB2 binding scFv identified (Table 7), we selected the one with the greatest binding to SKBR3 cells for further study (clone H7). The scFv gene was subcloned, scFv expressed and purified by IMAC. The H7 scFv was tested for SKBR3 growth inhibition in parallel with an irrelevant anti-botulinum scFv or with the 4D5 anti-ErbB2 mAb. We observed a strong inhibitory effect (50%) on cell growth using scFv H7 at a concentration of 300 nM (10 µg/ml). The extent of inhibition obtained was comparable to the maximal effect obtained using 4D5 and no inhibition was obtained with irrelevant scFv (figure 15A). To identify the H7 ligand (receptor bound), we first studied scFv H7 in a Western Blot experiment on SKBR3 cell lysates. Since no signal was detected, we used an alternative approach. We thus used scFv H7 to immunoprecipitate membrane biotinylated SKBR3 cell extracts. scFv H7 was captured on Ni-NTA agarose, and biotinylated cell extracts were incubated with the loaded agarose beads. Bound immunocomplexes were eluted using imidazole. The eluted fractions were run on an 8% SDS-PAGE, transferred onto nitrocellulose and blotted with a Streptavidin-HRP conjugate. We detected a major band running at 90 kD (p90). Using the same procedure, quantitative purification of p90 from native cell lysates was undertaken and submitted for N-terminal protein sequencing. The sequence corresponded to the N-terminal sequence of the transferrin receptor (TfR) (52). The identity of p90 as the receptor recognized by H7 was confirmed by blotting immunoprecipitates of SKBR3 lysates obtained with scFv H7 with a monoclonal anti-TfR. TfR has been described to be highly expressed in metabolically active cells such as tumor

cells and stem cells. Transferrin, the physiological ligand of the TfR, is a major carrier for iron and is rapidly internalized upon TfR binding. Following internalization, the iron is freed in vesicles and the receptor/ligand complex is recycled to the membrane. Some anti-TfR antibodies have been associated with a growth inhibitory effect (53, 54). To investigate the mechanism of the scFv H7 inhibitory effect on cell growth, we tested the effect of holotransferrin (iron charged transferrin) on the binding of scFv H7 phage to SKBR3 cells. Holotransferrin was able to inhibit scFv H7 phage binding to SKBR3 cells (IC_{50} 10 nM) (figure 15B,). Control experiments included inhibition of scFv H7-phage binding with soluble scFv H7 (IC_{50} 100 nM) and non inhibition of irrelevant phage (scFv F5 phage) binding by holotransferrin. We conclude that scFv H7 is an antagonist of transferrin. Its inhibitory effect on SKBR3 growth may result from the combined effects of inhibition of holotransferrin endocytosis and of down regulation of TfR from the cell surface leading to intracellular iron deprivation.

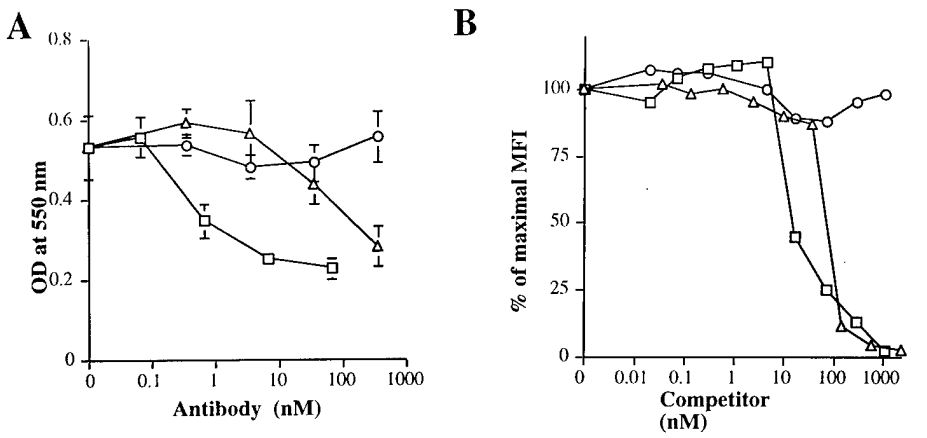

Figure 15. Growth inhibition of SKBR3 cells by scFv H7 and competition of H7 with holotransferrin for binding to the transferrin receptor. A. Growth inhibition of SKBR3 cells by H7 scFv. SKBR3 cells grown in 6-well plates were incubated for 5 days in the presence of increasing concentrations of (△) scFv-H7 , (□) 4D5 mAb or (○) irrelevant scFv-BotB . The number of living cells was estimated using the CellTiter growth assay kit (Promega) and reported as OD_{550}. B. Competition of H7 with holotransferrin for binding to the transferrin receptor. Inhibition of (□, △) H7 phage or (○) F5 phage binding to SKBR3 in the presence of increasing concentrations of holotransferrin (□, ○) or scFv-H7 (△). Bound phages were detected with anti-M13. Results are expressed in % of maximal mean fluorescent intensity (MFI), measured in the presence of no scFvs.

Significance: We have developed methodology and protocols which allow selection of antibodies from a phage library on the basis of endocytosis. This will be extremely valuable for targeted delivery of a wide variety of payloads to cells. In addition, since we used a monovalently displayed library, the antibodies are endocytosed as monomers, making construction of targeting molecules simpler. Furthermore, one of the two antibodies studied has a direct growth inhibitory effect on the selecting cell type (SKBR3).

2.4. Technical objectives 5 & 6: 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.

Years 1 through 3

Phage display has the potential to produce antibodies with affinities that cannot be produced using conventional hybridoma technology (24, 27-31). Ultra high affinity human antibody fragments could result in excellent tumor penetration, prolonged tumor retention, and rapid clearance from the circulation, leading to high specificity. We therefore undertook a series of experiments to produce ultra high affinity human antibody fragments. During the initial

years, experiments were performed to answer the following questions: 1) What is the most effective way to select and screen for rare higher affinity phage antibodies amidst a background of lower affinity binders; 2 What is the most effective means to remove bound phage from antigen, to ensure selection of the highest affinity phage antibodies; 3) What is the most efficient techniques for making mutant phage antibody libraries (random mutagenesis or site directed mutagenesis; 4) What region of the antibody molecule should be selected for mutagenesis to most efficiently increase antibody fragment affinity.

To answer these questions, we studied the human scFv C6.5, which binds the extracellular domain (ECD) of the tumor antigen ErbB-2 (32) with a K_d of 1.6×10^{-8} M and k_{off} of 6.3×10^{-3} s^{-1} (25). Isolation and characterization of C6.5 is described above. The isolation and initial characterization of C6.5 was partially supported by this grant, as well as by a subcontract to the Marks lab by National Cooperative Drug Discovery Group Group Award U01 CA 51880. Results of the experiments funded by this grant and described in prior reports and publications (27-31) provide a general approach for efficiently increasing antibody affinity and yielded human antibody fragments to the breast tumor antigen ErbB-2 with up to an 1200 fold increased affinity (K_d = 1.3×10^{-11} M). This represents the highest affinity tumor targeting antibody produced by any means. Our in vivo studies also demonstrate the importance of affinity and valency in specific tumor targeting (31, 32).

Significance: Protocols and methodologies have been developed which allow us to engineer antibody affinity to values higher than achievable from hybridoma technology. Higher affinity leads to increased tumor delivery of antibody.

2.5. Preclinical development of C6.5 and F5 based breast cancer therapies

Two approaches have been collaboratively pursued to develop C6.5 and F5 based breast cancer therapies. These are funded by other means than this grant, but since the antibodies were developed by funds from this grant, we report progress on this front. In collaboration with Dr. 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 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 antigen 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 ($t_{1/2}$ > 5 hours 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 tumor 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 CA65559-01.

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 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 hours, with 70% of the uptake internalized. The uptake is comparable to that previously reported using the 4D5 anti-HER2 Fab' from Genentech. There was no uptake of unconjugated liposomes. In vivo therapy studies in nude mice indicated that C6.5 targeted liposomes caused a significantly greater degree of tumor regression and cures than untargeted liposomes or a

combination of untargeted doxorubicin containing liposomes and systemic 4D5 Mab. At a December 1998 meeting of the National Cancer Institute Decision Network (DN), a decision was made to support C6.5 targeted doxorubicin containing liposomes at the 2A level. This means support for development of processes to increase antibody expression and scaleup for toxicology studies in anticipation of GMP manufacturing and a phase 1 clinical trial. One of the requests from the DN was that the hexahistidine epitope tag be removed from C6.5, necessitating a non-affinity purification technique. The resulting process resulted in only a 10% yield during purification. Fortuitously, F5 (see above) scFv binds protein A as it derived from the human VH3 family. Thus it can be purified without epitope tags with a yield of 90%. We therefore studied the liposomal targeting ability of F5 and the therapeutic effect of F5 targeted liposomes. Compared to untargeted doxorubicin containing liposomes, F5 yielded a statistically significantly greater reduction in tumor size (figure 16). A head to head comparison of C6.5 vs F5 targeted liposomes is ongoing. It is our anticipation that F5 will be as efficacious as C6.5 in targeting liposomes. If this is the case, than we will proceed with F5 for scaleup and clinical trial do to the ease of purification.

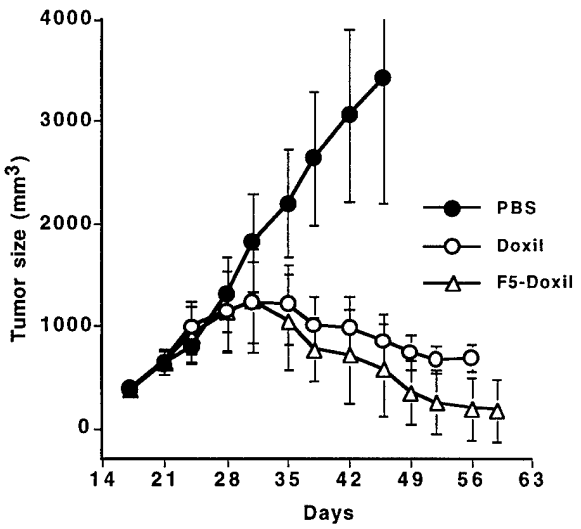

Figure 16. Mouse xenograft efficacy study of F5-doxil. Cohorts of 10-12 of nude mice with established subcutaneous xenografts of ErbB2-overexpressing human breast carcinoma (BT474) were injected with three weekly injections of F5-doxil and the tumor size monitored.

Significance: Phage antibody libraries, techniques and protocols have been developed which permit: 1) selection of human antibodies to any purified antigen; 2) the ability to engineer ultra high affinity human antibodies; and 3) the ability to select antibodies for endocytosis by cell surface receptors. This technology has been applied to produce ErbB2 binding scFv with novel properties. Compared to ErbB2 antibodies which are currently in clinical trials or approved for therapy of breast cancer, the antibodies are entirely human in sequence. In the case of C6.5, the scFv also bind with higher affinity and in the case of F5 are efficiently internalized as monomeric scFv. These unique features have led the University of California to file patent applications on both antibodies and on the method for selection of internalizing antibodies. We are currently pursuing two 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

3.1. A large (7.0×10^9 member) scFv 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. This library is a potential source of

unique human antibodies to relevant breast tumor antigens. (Addresses technical objectives 1 and 2).

3.2 Phage antibodies binding cell surface receptors can be efficiently internalized by mammalian cells and recovered in an infectious state from within the cell. This approach allows enrichment of internalizing antibodies more than 1000 fold over non-internalizing antibodies and provides a general method for directly selecting phage libraries on cells to isolate cell type specific antibodies. This approach is much more efficient with respect to enrichment ration than recovering phage from the cell surface.

3.3 The 'selection for internalization' methodology can be applied to a library yielding breast tumor specific scFv. Using this approach, we isolated rapidly internalizing scFv to ErbB2 and the transferrin receptor. Using one of these scFv, it was possible to immunoprecipitate and sequence the receptor bound, providing a method for identification of the receptor recognized by the internalizing phage antibodies. Many more scFv, that appear to be tumor specific, remain to be characterized. This should be an important approach for identifying tumor specific antigens and antibodies. (Points 3.2 through 3.3 address technical objectives 3 and 4).

3.4 We have developed methods for selection of antibodies on purified antigen which led to the isolation of a unique human scFv that binds the breast tumor antigen ErbB2 (C6.5). Methodologies were developed that provide a general route to increasing antibody affinity to values not previously reported and higher than achievable using hybridoma technology. (Addresses technical objectives 5 and 6).

3.5 During the coming year, we will characterize additional internalizing phage antibodies with respect to specificity and antigen recognized. We will attempt to identify antigens which are tumor specific and heretofore unknown.

3.6. The anti-ErbB2 antibodies generated from this work have been shown to specifically target ErbB2 breast tumor cells *in vivo* and are being developed as targeting agents for doxorubicin containing liposomes with plans for an NCI sponsored phase 1 clinical trial.

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Appendix 1

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Cell Biology

Efficient construction of a large nonimmune phage antibody library: The production of high-affinity human single-chain antibodies to protein antigens

(single-chain Fv/phage display/antibody libraries/human mAbs)

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Contributed by John C. Gerhart, March 16, 1998

ABSTRACT **A large library of phage-displayed human single-chain Fv antibodies (scFv), containing 6.7×10^9 members, was generated by improving the steps of library construction. Fourteen different protein antigens were used to affinity select antibodies from this library. A panel of specific antibodies was isolated with each antigen, and each panel contained an average of 8.7 different scFv. Measurements of antibody–antigen interactions revealed several affinities below 1 nM, comparable to affinities observed during the secondary murine immune response. In particular, four different scFv recognizing the ErbB2 protein had affinities ranging from 220 pM to 4 nM. Antibodies derived from the library proved to be useful reagents for immunoassays. For example, antibodies generated to the *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 mAbs to a wide variety of protein antigens. Such libraries should prove especially useful for generating reagents to study the function of gene products identified by genome projects.**

Antibodies that bind with high specificity and high affinity to a target molecule are essential tools for biological research. These reagents have proven invaluable for: (*i*) detecting and quantitating levels of gene expression; (*ii*) determining the subcellular, cellular, and tissue location of gene expression; and (*iii*) 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 Ig variable (V) region genes (1–3). Single-chain Fv antibodies (scFv) have proven particularly useful. scFv consist of the antigen-binding domains of Ig 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 (6–8). 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.

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, hybridoma technology, a slow and cumbersome process, was used to produce mAbs for such applications. 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.

Recent advances using antibody phage display now make it possible to overcome these limitations and generate human mAbs that recognize any desired antigen (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 nonbinding phage antibodies with affinity chromatography techniques (12–15). This strategy requires no immunization, the antibody genes are cloned, and generally the antibody fragments express well in *Escherichia 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 because library diversity and utility are lost on library reamplification.

In this paper, we describe a strategy to optimize the construction of phage-display antibody libraries. By using this strategy, a very large phage-displayed single-chain antibody library consisting of 6.7×10^9 members was produced. This library then was used to isolate panels of antibodies to 14 different protein antigens. Analysis of antibody–antigen interactions revealed high-affinity binding with K_ds for the ErbB2 protein ranging between 220 pM and 4 nM.

METHODS

Construction of the V_H Library. Total RNA was prepared from three different samples of human spleen cells and two

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Abbreviations: BMP, bone morphogenetic protein; BoNT, botulinum neurotoxin; ECD, extracellular domain; CDR, complementarity determining region; EB, elementary body; scFv, single-chain Fv fragment; V_κ, Ig kappa light chain variable region; V_λ, Ig lambda light chain variable region; V_L, Ig light chain variable region; V_H, Ig heavy chain variable region.

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different samples of human peripheral blood lymphocytes. cDNA was synthesized from total RNA primed with the HuIgMFOR primer (12). V_H gene repertoires were amplified from the cDNA by 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 and reamplified to append *Nco*I and *Not*I restriction sites by using Tth DNA polymerase (Epicentre Technologies, Madison, WI) and an equimolar mixture of the HuVHBACKSfi primers (that contain an *Nco*I site for cloning) and the HuCMForNot primer (5′-GAGTC-ATTCTCGACTTGCGGCCGCTGGAAGAGGCACGTTC-TTTTCTTT-3′) (12). The PCR products were cut with restriction enzymes *Nco*I and *Not*I and agarose gel-purified. The resulting DNA fragments were ligated into the plasmid pCITE3A (Novagen) cut with restriction enzymes *Nco*I and *Not*I and the ligated DNA was electroporated into the *E. coli* strain TG1. A library of V_H genes containing 2.3×10^8 members was generated from the products of seven ligation reactions and 15 electroporations. The resulting library was termed pCITE-V_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 because of the presence of unique sequences for PCR amplification that surround the *Nco*I and *Not*I cloning sites. These sequences allow the specific amplification of the V_H genes for scFv assembly. This strategy is advantageous for amplification of the V_H genes and also the subsequent amplification of scFv genes assembled from the 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.

Construction of the scFv Library. The V_H gene repertoire was PCR-amplified from the pCITE-V_H library by using 300 ng of library plasmid DNA as a template, Vent DNA polymerase, the CITE3 primer (5′-GATCTGATCTGGGGCCTCGGT-GC-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 $(G_4S)_3$ (4), was amplified from 300 ng of library plasmid DNA by using Vent DNA polymerase, the Gene3 primer (5′-GC-AAGCCCAATAGGAACCCATGTACCG-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 with overlap extension PCR, the input DNA fragments must have blunt ends. Therefore, the proofreading DNA polymerase Vent was used to generate the V_H and V_L DNA fragments for scFv assembly. For all subsequent PCR steps of library construction Tth DNA polymerase was found to be the optimal enzyme. The V_H and V_L gene repertoires were spliced together in 100- μl PCRs containing 100 ng of the V_H and V_L DNA fragments and Tth DNA polymerase. The reactions were cycled eight 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 was cycled 30 times (94°C 1 min, 55°C 1 min, and 72°C 3 min) 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, personal communication), activin receptor type II (18), bone morphogenetic protein (BMP) receptor type I (19, 20), and fibroblast growth factor receptor (21) were cloned into pMAL expression plasmids as fusions with the gene encoding maltose binding protein 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 (Univ. of California, Berkeley), and BoNT/B, C, and E were provided by Theresa Smith (United States Army Medical Research Institute of Infectious Disease). BoNT/A C-fragment was purchased from Ophidian (Madison, WI). Human ErbB-2 extracellular domain (ECD) was provided by James Huston (Creative Biomolecules) (22), human cytochrome b5 was provided by by Lucy Waskell (Univ. of California, San Franscisco), and human vascular endothelial growth factor was provided by James Hoeffler (Invitrogen).

Selection of Phage Antibodies. Phagemid particles were rescued from the library, as described (23) except that the procedure was scaled up to 2 liters of culture media. Specific phage-displayed scFv were affinity-selected by using proteins absorbed to Immunotubes (Nunc) (12). For selections with maltose binding protein (MBP) fusion proteins, phage were preincubated with 50 μg of purified MBP to deplete the library of MBP antibodies. For selection of scFv to the Erb-B2 ECD, Immunotube selection was alternated with selection using decreasing concentrations of biotinylated Erb-B2 ECD and

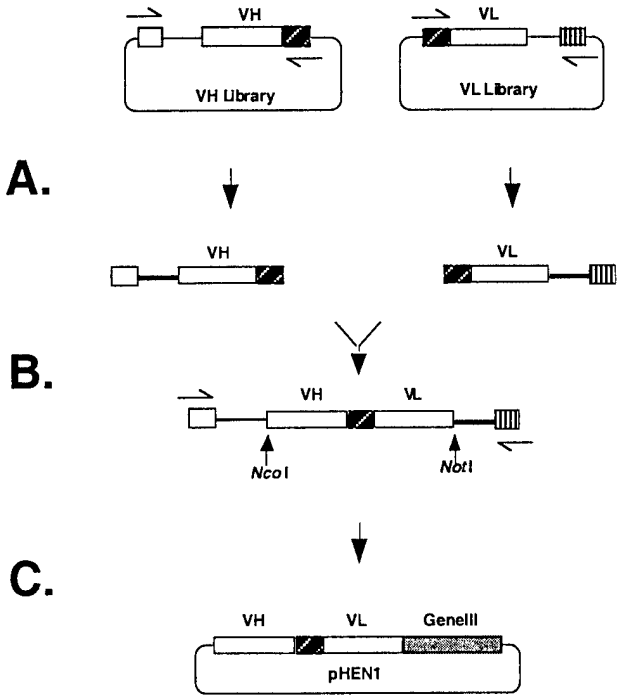

FIG. 1. Schematic outline of the approach used for library construction. A library of V_H and genes was generated from rearranged human V-genes and cloned into the plasmid pCITE3A. The V_L genes used for scFv assembly were derived from a previously constructed scFv library contained in the plasmid pHEN1 (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 bp 5′ (the V_H genes) or 3′ (the V_L genes) of the scFv gene cloning sites. This approach 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 wereamplified by using a plasmid specific primer ( ) and an equimolar mixture of HuJ_H primers ( ). The linker DNA and V_L genes were amplified by using a plasmid specific primer ( )and an equimolar mixture of $RHuJ_H$ primers ( ). The RHuJH primers are complementary to the HuJ_H primers. (*B*) The V_H and linker DNA-V_L gene repertoires were PCR assembled into a scFv gene repertoire. (*C*) The assembled scFv gene repertoire was cut with the restriction enzymes *Nco*I and *Not*I and cloned into the plasmid pHEN1 (17) for phage display.

capture of bound phage using streptavidin paramagnetic beads (23). For selection of scFv that bind *Chlamydia* antigens, Immunotubes were coated overnight at room temperature with 1 ml of *C. trachomatis* strain L2/434/Bu elementary bodies (EBs) at a concentration of 0.1 mg/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 3–4 times, after which individual clones were analyzed for specific antigen binding by ELISA.

Antibody Binding Specificity. The binding specificity of all scFv was determined by ELISA using the target antigen and at least nine other proteins as substrates (12). The number of unique scFv was estimated by PCR fingerprinting of the scFv genes with the restriction enzyme *Bst*NI and confirmed by DNA sequencing (12, 16). Putative V_H and V_L germ-line gene segment derivation was determined with 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 by 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. Two hundred microliters of *C. trachomatis* EBs at 8×10^6 inclusion forming units/μl were used to infect the monolayers (28). The infected cells were incubated for 48 hr at 37°C, washed with PBS, and fixed with 100% methanol for 10 min. Purified scFv (50 μg/ml) was incubated with fixed cells for 1 hr at room temperature. scFv binding was detected with the 9E10 mAb that recognizes the c-myc epitope present in the scFv (29) (1 μg/ml) followed by fluorescein isothiocyanate-conjugated anti-mouse Fc (Zymed). Cells were counterstained with Evans blue and visualized with fluorescence microscopy.

RESULTS

Library Construction. A very large phage antibody library was created for the routine isolation of high-affinity scFv antibodies to any target protein. This library was generated by optimizing the individual steps of library construction to increase the efficiency of scFv gene assembly and increase the efficiency of cloning scFv genes (Fig. 1). First, scFv antibodies were assembled from cloned V_H and V_L gene repertoires contained in separate plasmid vectors. A library of V_H genes, containing 2.3×10^8 members, was specifically created for generating an additional scFv repertoire. The V_L genes for scFv assembly were derived from an existing scFv repertoire containing 3.0×10^7 members (Fig. 1*A*). The use of cloned libraries as a source of V-genes provided a stable and limitless supply of material for scFv assembly. For the construction of previous antibody libraries, scFv gene repertoires were directly assembled from V_H and V_L reverse transcription–PCR (RT-PCR) products (12). With this previous approach, RNA availability and the efficiency of RT-PCR limited the quantity of V-genes available for scFv construction. 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. 1*B*). Using V_L genes already fused to the peptide linker allowed us to construct scFv from only two DNA fragments. Previously, scFv gene repertoires were inefficiently assembled from three separate DNA fragments consisting of V_H and V_L gene repertoires and linker DNA (12). Third, the V_H and V_L gene repertiores and the scFv genes assembled from these repertoires were amplified with primers that annealed to sequences approximately 200 bp 5′ of the V_H genes and to sequences approximately 200 bp 3′ of the V_L genes. This strategy generated long sequence extensions at the ends of the individual V_H, V_L gene segments, and the assembled scFv. These sequence extensions ensured efficient cutting with the restriction enzymes *Nco*I and *Not*I that were used for scFv cloning and facilitated the identification of the correctly assembled scFv (Fig. 1*C*).

By 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

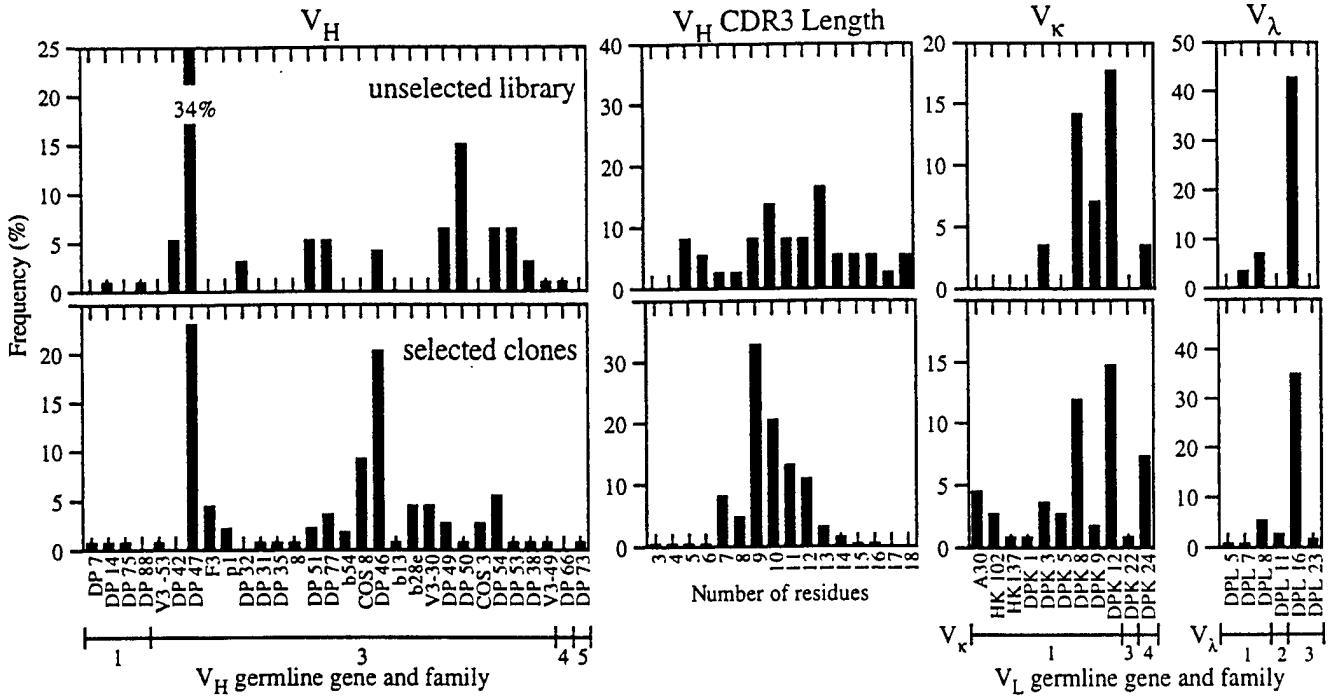

FIG. 2. V-gene usage and V_H CDR3 length of unselected and antigen-specific scFv. The V_H and V_L genes were sequenced and the germ-line gene was assigned based on homology to a database (VBASE) of germ-line V-genes compiled by Tomlinson *et al.* (25). Specific V_H, V_κ, and V_λ genes are listed on the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

Table 1. Results of phage antibody library selections

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
C. trachomatis EB	66 (63/96)	7

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. FGF, fibroblast growth factor; VEGF, vascular endothelial growth factor.

randomly chosen scFv revealed 36 unique sequences and a relatively random distribution of V_H complementarity determining region (CDR) 3 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, $V_\kappa1$, and $V_\lambda3$) 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 also may be caused by differences in 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 by using 13 different purified protein antigens from a variety of species, including human and EBs from *C. trachomatis* (Table 1). Given our interest in developmental biology, four of these proteins were the extracellular domains of different *Xenopus* growth factor receptors: the activin receptor types I and II, the BMP receptor type I, and the fibroblast growth factor receptor (19–21). After at least three rounds of selection with a particular antigen, the binding specificity of individual scFv was 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 first were characterized by DNA fingerprinting (12, 16) and then DNA sequencing (23). This analysis revealed 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). Because only a small number of clones from each selection were analyzed, it is likely that screening of more clones 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 *C. trachomatis*-specific scFv stains *C. trachomatis* elementary bodies within infected cells while neighboring uninfected cells remain unstained.

V-gene derivation of scFv antibodies that bound to the different antigens was diverse (Fig. 2). V_H genes were derived from three of the six V_H gene families (nos. 1, 3, and 5) and

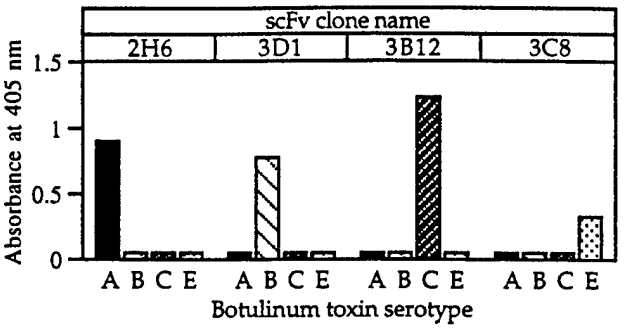

FIG. 3. Specificity of anti-Botulinum neurotoxin scFv. Representative scFv (2H6, 3D1, 3B12, and 3C8) isolated respectively from selections on BoNT serotypes A, B, C, and E were studied. Specificity was determined by ELISA.

from 26 different germ-line genes. V_L genes were derived from three of the six V_κ gene families (nos. 1, 3, and 4) and 11 different V_κ germ-line genes, from three of the nine V_λ gene families (nos. 1–3), and nine different V_λ germ-line genes. Despite the diversity, there was a bias seen in the V-gene usage. V_H genes largely were derived from the V_H3 family, particularly DP46 and DP47. V_κ genes most frequently were derived from the $V_\kappa1$ family while V_λ genes most frequently were derived from the $V_\lambda3$ 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 also may reflect expression biases of *E. coli*. The number of sequenced V-genes from previous nonimmune 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 were calculated from association and dissociation rate constants measured by using surface plasmon resonance (Table 2) (23, 27). The K_d of the antibodies ranged from 220 pM to 4 nM for anti-ErbB-2 scFv and 38 nM to 71 nM for anti-BoNT/A scFv. The affinity of the

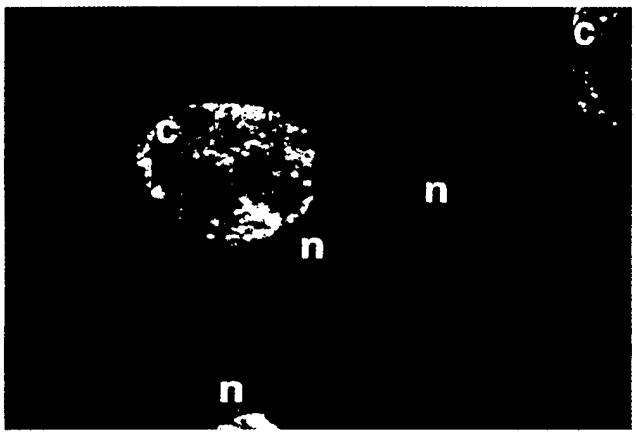

FIG. 4. Staining of HeLa cells infected with *C. trachomatis* with the scFv 2A10. The scFv specifically stains *C. trachomatis* elementary bodies (c) within infected HeLa cells but does not stain uninfected cells. n = nucleus.

Table 2. Affinities and binding kinetics of anti-BoNT A C-fragment and anti-Erb-B2 scFv

Specificity and clone	K_d ($\times 10^{-9}$ M)	k_{on} ($\times 10^5$ $M^{-1}s^{-1}$)	k_{off} ($\times 10^{-3}$ 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

Association (k_{on}) and dissociation (k_{off}) rate constants for purified scFv were measured by using surface plasmon resonance (BIAcore) and K_d was calculated as (k_{off}/k_{on}).

anti-ErbB-2 scFv B7A is the highest observed for any antibodies isolated from nonimmune phage antibody libraries (14, 15). The affinities of the isolated scFv are also comparable to affinities of mAbs derived from the secondary immune response (38).

The different K_ds observed for scFv that bind ErbB-2 and BoNT/A are 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 the concentration of antigen used for selection and 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. By 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, by 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 mAbs from the secondary murine immune response (Table 2). In addition, these antibody antigen binding affinities are the highest reported for antibodies from nonimmune phage antibody libraries (12, 14, 15). Fifth, isolated scFv served as functional reagents in a number of different immunoassays including ELISA, immunofluorescence (Figs. 3 and 4), Western blotting, epitope mapping, and immunoprecipitation (data not shown).

Nonimmune 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 Ig mRNA as the source of V-genes ensures that close to 100% of the gene sequences will be functionally rearranged with ORFs (results from this work and ref. 34). Fewer V-genes will have an ORF 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 nonimmune 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*.

Nonimmune phage antibody libraries already are 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 and elucidate the function of specific molecules *in vivo* (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 investigator's imagination.

Table 3. Comparison of protein binding antibodies selected from nonimmune phage-display antibody libraries

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 ($\times 10^{-9}$ 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	100–2,500
Griffiths *et al.* (14)	6.5×10^{10}(Fab, SS)	30	4.8	3	7.0–58
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

*For library type, N = V-gene repertoires obtained from V-genes rearranged *in vivo*; SS = semisynthetic V-genes constructed from cloned V-gene segments and synthetic oligonucleotides encoding V_H CDR3. ND, not determined.

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Appendix 2

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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 $(scFv\text{-}C_H3)_2$ 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), 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.

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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

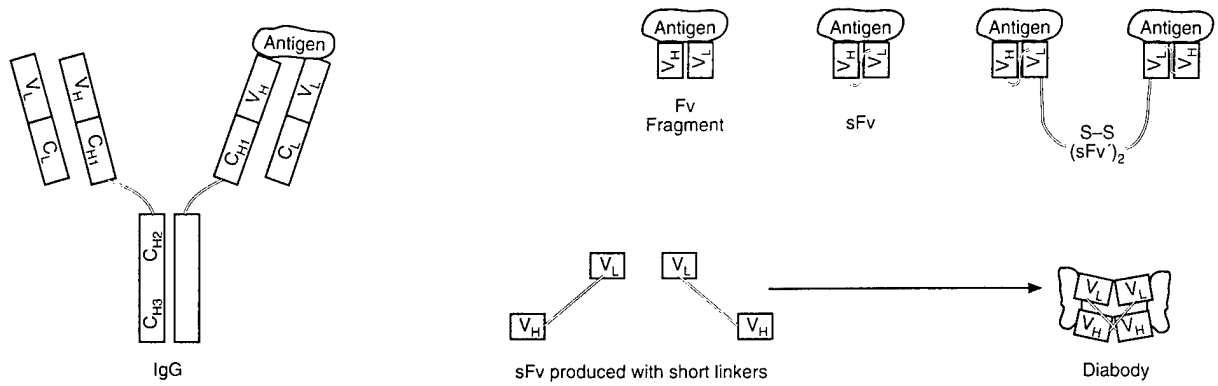

Figure 1 The structures of an IgG molecule (~150 kDa), an Fv fragment (~22 kDa), a recombinant scFv (~25 kDa) molecule, a dimeric $(scFv')_2$ molecule (~55 kDa) and a diabody molecule (~50 kDa) are presented. While they are identical, the two diabody components are illustrated with different shading 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 *Nco*I and *Not*I, gel purified and ligated into *Nco*I/*Not*I-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 $3.2–4.8 \times 10^3$ 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).

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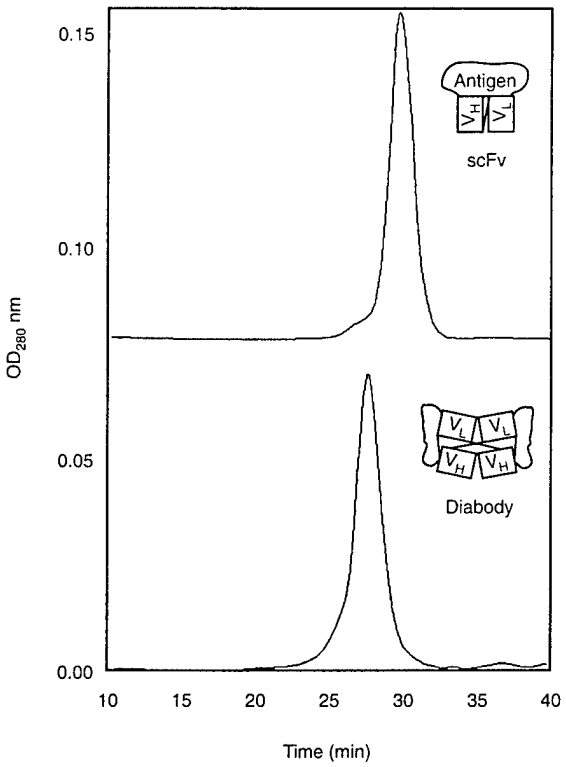

Figure 2 HPLC profiles of the C6.5 diabody and C6.5 scFv. After IMAC purification on a Ni^{++} agarose column, the C6.5 diabody and scFv were analysed on a Superdex 200 column (Pharmacia). The C6.5 scFv eluted from the Superdex 200 column as a single peak of approximately 25 kDa with minimal evidence 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

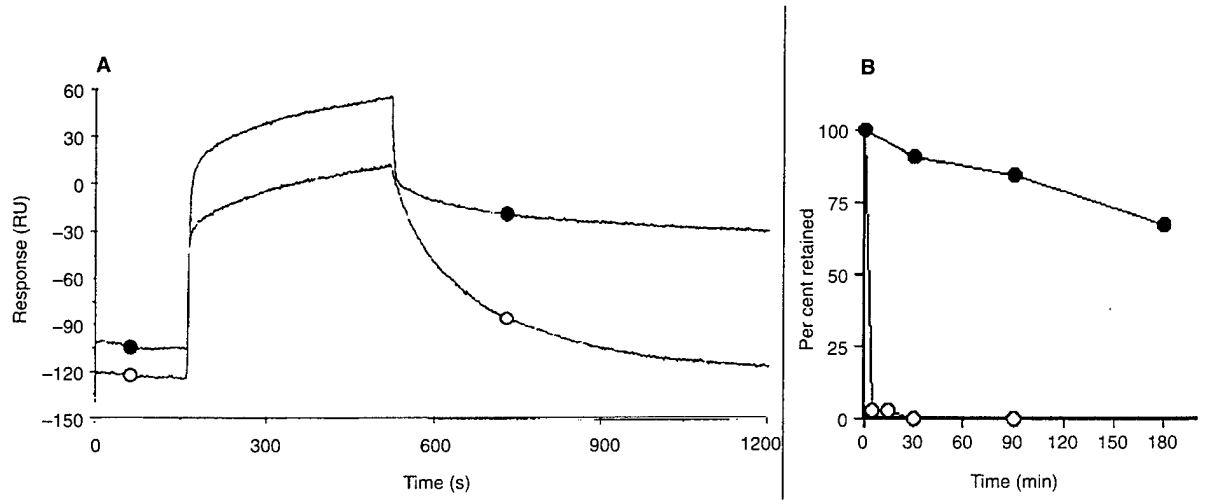

Figure 3 In vitro characterization of the C6.5 diabody. C6.5 diabody and monomeric scFv were evaluated by surface plasmon resonance (BIAcore) as described in Methods. The association and dissociation kinetics are displayed for the diabody (●) and scFv monomer (○) forms of C6.5 (**A**). In vitro cell-surface 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**)

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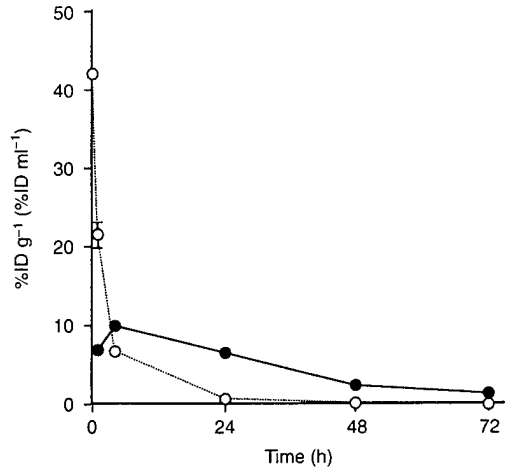

Figure 4 The in vivo tumour targeting of radioiodinated C6.5 diabody was determined in a biodistribution study performed in SK-OV-3 tumour-bearing *scid* mice. The plotted values represent the mean tumour (●) and blood (○) retentions obtained from six mice per data point. The standard errors are indicated

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 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

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.

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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 $\leq$ 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 (RAIT) 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.

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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 $(scFv\text{-}CH3)_2$ 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 radioimmunoimaging 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 $(scFv\text{-}C_H3)_2$ 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

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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-dimethylthiazol-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 3

Towards selection of internalizing antibodies from phage libraries

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Abstract

Antibodies which bind cell surface receptors in a manner whereby they are endocytosed are useful molecules for the delivery of drugs, toxins or DNA into the cytosol of mammalian cells for therapeutic applications. Traditionally, internalizing antibodies have been identified by screening hybridomas. For this work, we studied a human scFv (C6.5) which binds ErbB2 to determine the feasibility of directly selecting internalizing antibodies from phage libraries and to identify the most efficient display format. Using wild type C6.5 scFv displayed monovalently on a phagemid, we demonstrate that anti-ErbB2 phage antibodies can undergo receptor mediated endocytosis. Using affinity mutants and dimeric diabodies of C6.5 displayed as either single copies on a phagemid or multiple copies on phage, we define the role of affinity, valency, and display format on phage endocytosis and identify the factors that lead to the greatest enrichment for internalization. Phage displaying bivalent diabodies or multiple copies of scFv were more efficiently endocytosed than phage displaying monomeric scFv and recovery of infectious phage was increased by preincubation of cells with chloroquine. Measurement of phage recovery from within the cytosol as a function of applied phage titer indicates that it is possible to select for endocytosable antibodies, even at the low concentrations that would exist for a single phage antibody member in a library of 10^9.

Introduction

Growth factor receptors are frequently overexpressed in human carcinomas and other diseases and thus have been utilized for the development of targeted therapeutics. The *HER2/neu* gene, for example, is amplified in several types of human adenocarcinomas, especially in tumors of the breast and the ovary (1) leading to the overexpression of the corresponding growth factor receptor ErbB2. Targeting of ErbB2 overexpressing cells has been accomplished primarily using anti-ErbB2 antibodies in different formats, including conjugation to liposomes containing chemotherapeutics (2), fusion to DNA carrier proteins delivering a toxic gene (3), and direct fusion to a toxin (4). For many of these targeted approaches, it is necessary to deliver the effector molecule across the cell membrane and into the cytosol. This can be accomplished by taking advantage of normal growth factor receptor biology; growth factor binding causes receptor activation via homo- or heterodimerization, either directly for bivalent ligand or by causing a conformational change in the receptor for monovalent ligand, and receptor mediated endocytosis (5). Antibodies can mimic this process, stimulate endocytosis, become internalized and deliver their payload into the cytosol. In general, this requires a bivalent antibody capable of mediating receptor dimerization (6, 7). In addition, the efficiency with which antibodies mediate internalization differs significantly depending on the epitope recognized (7, 8). Thus for some applications, such as liposomal targeting, only antibodies which bind specific epitopes are rapidly internalized and yield a functional targeting vehicle.

Currently, antibodies which mediate internalization are identified by screening hybridomas. Alternatively, it might be possible to directly select internalizing antibodies from large non-immune phage libraries (9, 10) by recovering infectious phage particles from within cells after receptor mediated endocytosis, as reported for peptide phage libraries (11, 12). Unlike the multivalently displayed peptide phage libraries, however, phage antibody libraries typically display monomeric single chain Fv (scFv) or Fab antibody fragments fused to pIII as single copies on the phage surface using a phagemid system (9, 10). We hypothesized that such monovalent display was unlikely to lead to efficient receptor crosslinking and phage internalization. To determine the

feasibility of selecting internalizing antibodies and to identify the most efficient display format, we studied a human scFv (C6.5) which binds ErbB2 (13). Using wild type C6.5 scFv, we demonstrate that anti-ErbB2 phage antibodies can undergo receptor mediated endocytosis. Using affinity mutants and dimeric diabodies of C6.5 displayed as either single or multiple copies on the phage surface, we define the role of affinity, valency, and display format on phage endocytosis and identify the factors that lead to the greatest enrichment for internalization. The results indicate that it is possible to select for endocytosable antibodies, even at the low concentrations that would exist for a single phage antibody member in a library of 10^9 members.

Material and methods

Cells

The SKBR3 breast tumor cell line was obtained from ATCC and grown in RPMI media supplemented with 10% FCS (Hyclone) in 5% CO_2 at 37°C.

Antibodies and antibody phage preparations

The C6.5 scFv phage vector was constructed by subcloning the C6.5 gene as a *Sfi* I/*Not* I fragment from scFv C6.5 pHEN1 (13) into the phage vector fd/*Sfi* I/*Not* I (a gift of Andrew Griffiths, MRC Cambridge, UK). The C6.5 diabody phagemid vector was constructed by subcloning the C6.5 diabody gene (14) as a NcoI/NotI fragment into pHEN1 (15). The anti-botulinum scFv phagemid (clone 3D12) (16) C6.5 scFv phagemid (13) and scFv C6ML3-9 scFv phagemid (17) in pHEN1 have been previously described. Phage were prepared (18) from the appropriate vectors and titered on *E. coli* TG1 as previously described (9) using ampicillin (100 μg/ml) resistance for titration of constructs in pHEN1 and tetracyline (50 μg/ml) for titration of constructs in fd. Soluble C6.5 scFv, C6.5 diabody and anti-botulinum scFv were expressed from the vector pUC119mycHis (13) and purified by immobilized metal affinity chromatography as described elsewhere (13).

Detection of internalized native antibody fragments and phage antibodies

SKBR3 cells were grown on coverslips in 6-well culture plates (Falcon) to 50% of confluency. Culture medium was renewed 2 hours prior to the addition of 5.10^{11} cfu/ml of phage preparation (the phage preparation representing a maximum of 1/10 of the culture medium volume) or 20 µg/ml of purified scFv or diabody in phosphate buffered saline, pH 7.4 (PBS). After 2 hours of incubation at 37°C, the wells were quickly washed 6 times with ice cold PBS and 3 times for 10 minutes each with 4 mL of stripping buffer (50 mM glycine pH 2.8, 0.5 M NaCl, 2M urea, 2% polyvinylpyrrolidone) at RT. After 2 additional PBS washes, the cells were fixed in 4% paraformaldehyde (10 minutes at RT), washed with PBS, permeabilized with acetone at -20°C (30 seconds) and washed again with PBS. The coverslips were saturated with PBS-1% BSA (20 min. at RT). Phage particles were detected with biotinylated anti-M13 immunoglobulins (5 Prime-3 Prime, Inc, diluted 300 times) (45 min. at RT) and Texas red-conjugated streptavidin (Amersham, diluted 300 times) (20 min. at RT). Soluble scFv and diabodies containing a C-terminal myc peptide tag were detected with the mouse mAb 9E10 (Santa Cruz Biotech, diluted 100 times) (45 min. at RT), anti-mouse biotinylated immunoglobulins (Amersham, diluted 100 times) and Texas red-conjugated streptavidin. Optical confocal sections were taken using a Bio-Rad MRC 1024 scanning laser confocal microscope. Alternatively, slides were analyzed with a Zeiss Axioskop UV fluorescent microscope.

Recovery and titration of cell surface bound or internalized phage

Subconfluent SKBR3 cells were grown in 6-well plates. Culture medium was renewed 2 hours prior to the experiment. Cells were incubated for varying times with different concentrations of phage preparation at 37°C (specific details for each experiment are provided in the Table or figure legends). Following PBS and stripping buffer washes, performed exactly as described above for detection of internalized native antibody fragments and phage antibodies, the cells were washed again twice with PBS and lysed with 1 mL of 100 mM triethylamine (TEA). The stripping

buffer washes and the TEA lysate were neutralized with 1/2 volume of Tris-HCl 1M, pH 7.4. For some experiments (see figure legends for specifics), cells were trypsinized after the three stripping buffer washes, collected in a 15 ml Falcon tube, washed twice with PBS and then lysed with TEA. In experiments performed in the presence of chloroquine, SKBR3 cells were preincubated for two hours in the presence of complete medium containing 50 μM chloroquine prior to the addition of phage. Corresponding control samples in the absence of chloroquine were prepared at the same time. For all experiments, phage were titered on *E. coli* TG1 as described above.

Results

1) The model system utilized to study phage antibody internalization

The human anti-ErbB2 scFv C6.5 was obtained by selecting a human scFv phage antibody library on recombinant ErbB2 extracellular domain (13). C6.5 scFv binds ErbB2 with a K_d = 1.6 x 10^{-8} M and is a stable monomeric scFv in solution with no tendency to spontaneously dimerize or aggregate (13). To determine the impact of affinity on internalization, we studied a scFv (C6ML3-9) which differs from C6.5 by 3 amino acids (17). C6ML3-9 scFv is also a stable monomer in solution and binds the same epitope as C6.5 scFv but with a 16 fold lower K_d (1.0 x 10^{-9} M) (17, 19). Since receptor homodimerization appears to typically be requisite for antibody internalization we also studied the dimeric C6.5 diabody (14). Diabodies are scFv dimers where each chain consists of heavy (V_H) and light (V_L) chain variable domains connected using a peptide linker which is too short to permit pairing between domains on the same chain. Consequently, pairing occurs between complementary domains of two different chains, creating a stable noncovalent dimer with two binding sites (20). The C6.5 diabody was constructed by shortening the peptide linker between the Ig V_H and V_L domains from 15 to 5 amino acids and binds ErbB2 on SKBR3 cells bivalently with a K_d approximately 40 fold lower than C6.5 (4.0 x 10^{-10} M) (14).

Native C6.5 scFv and C6.5 diabody was expressed and purified from *E. coli* and analyzed for endocytosis into ErbB2 expressing SKBR3 breast tumor cells by immunofluorescent confocal

microscopy. As expected, monomeric C6.5 scFv is not significantly internalized whereas the dimeric C6.5 diabody can be detected in the cytoplasm of all cells visualized (figure 1).

For subsequent experiments, the C6.5 and C6ML3-9 scFv and C6.5 diabody genes were subcloned for expression as pIII fusions in the phagemid pHEN-1 (15). This should yield phagemid predominantly expressing a single scFv or diabody-pIII fusion after rescue with helper phage (21) (figure 2A and B). Diabody phagemid display a bivalent antibody fragment resulting from intermolecular pairing of one scFv-pIII fusion molecule and one native scFv molecule (figure 2B). The C6.5 scFv gene was also subcloned into the phage vector fd-Sfi/Not. This results in phage with 3 to 5 copies each of scFv-pIII fusion protein (figure 2C). The human breast cancer cell line SKBR3 was used as a target cell line for endocytosis. Its surface ErbB2 density is approximately 1.0×10^6 per cell (22).

2) C6.5 phagemids are endocytosed by human cells

C6.5 scFv phagemids were incubated for 2 hours with SKBR3 cells grown on coverslips at 37°C to allow active internalization. Cells were extensively washed with PBS to remove non specific binding and washed an additional three times with high salt and low pH (stripping) buffer to remove phage specifically bound to cell surface receptors. Internalized phagemid were detected with a biotinylated M13 antiserum recognizing the major coat phage protein pVIII. An anti-botulinum toxin phagemid was used as a negative control. Staining was analyzed by using immunofluorescent microscopy (Figure 3). Approximately 1% of the cells incubated with C6.5 scFv phagemid showed a strong intracellular staining consistent with endosomal localization (figure 3B) while no staining was observed for anti-botulinum phagemid (figure 3A). Furthermore, no staining was seen if the incubation was performed for 2 hours at 4°C instead of 37°C (data not shown). Staining performed after the PBS washes but before washing with stripping buffer showed membrane staining of all the cells, indicating that multiple washes with stripping buffer is necessary to remove surface bound phagemids. The results also indicate that only a fraction of the cell bound phage are endocytosed.

3) Increased affinity and bivalency lead to increased phage endocytosis

We compared the internalization of C6.5 scFv, C6ML3-9 scFv and C6.5 diabody phagemid and C6.5 scFv phage using immunofluorescence. Both C6ML3-9 scFv and C6.5 diabody phagemid as well as C6.5 scFv phage yielded increased intensity of immunofluorescence observed at the cell surface compared to C6.5 scFv phagemid. For C6ML3-9 scFv phagemid, approximately 10% of the cells showed intracellular fluorescence after 2 hours of incubation (Figure 3C). This value increased to approximately 30% of cells for the dimeric C6.5 diabody phagemid (figure 3D) and 100% of cells for multivalent C6.5 scFv phage (figure 3E).

4) Infectious phage can be recovered from within the cell and their titre correlates with the level of uptake observed using immunofluorescence

To determine if infectious phage antibody particles could be recovered from within the cell, we incubated approximately 5.0 x 10^5 SKBR-3 cells for 2 hours at 37°C with 3.0 x 10^{11} cfu of the different phagemid or phage. Six PBS washes were used to remove non-specifically bound phage and specifically bound phage were removed from the cell surface by three consecutive washes with stripping buffer (washes I, II and III respectively, Table 1) . The cells were then lysed with 1 mL of a 100 mM triethylamine solution (TEA) (representing the intracellular phage). The three stripping washes and the cell lysate were neutralized and their phage titer was determined by infection of *E. coli* TG1. The titers of phage recovery are reported in Table 1.

Considerable background binding was observed in the first stripping wash for the anti-botulinum phage even after 6 PBS washes (2.8 x 10^7 cfu, Table 1). This value likely represents phage non-specifically bound to the cell surface as well as phage trapped in the extracellular matrix. The amount of surface bound phage increased only 2.1 fold above this background for C6.5 scFv phagemid (Tables 1 and 2). With increasing affinity and avidity of the displayed C6.5 antibody fragment, the titer of cell surface bound phagemid or phage increased (Table 1). The titer of phage in the consecutive stripping washes decreased approximately 10 fold with each wash. These

additional stripping washes led to a minor increase in the titer of specific phage eluted compared to the background binding of the anti-botulinum phage (2.7 fold for C6.5 scFv phagemid to 20 fold for C6.5 scFv phage, Table 2). The only exception was the titer of the C6.5 diabody phagemid, where the ratio actually decreased from 6.4 fold to 4.6 fold. This is likely due to the fact that in the diabody the V_H and V_L domains that comprise a single binding site are not covalently attached to each other via the peptide linker. This increases the likelihood that a stringent eluent (like glycine) could dissociate V_H from V_L and abrogate binding to antigen.

Three stripping washes were required to ensure that the titer of phage recovered after cell lysis was greater than the titer in the last stripping wash (Table 1). We presumed that after three stripping washes, the majority of the phage eluted represented infectious particles from within the cell rather than from the cell surface. In fact, since the cell lysate titer observed with non-specific anti-botulinum phage was considerable (1.5×10^6) and greater than observed in the last stripping wash, it is likely that many phage remain trapped within the extracellular matrix and relatively inaccessible to the stripping buffer washes. Some anti-botulinum phage might also be non-specifically endocytosed by cells, but this is likely to be a small amount given the immunofluorescence results (figure 3). The titer of phage in the TEA fraction increased with increasing affinity and avidity of C6.5, with the highest titers observed for the dimeric C6.5 diabody phagemid and the multivalent C6.5 scFv phage (Table 1). The values represent a 30 fold (C6.5 diabody phagemid) and 146 fold (C6.5 scFv phage) increase in titer compared to the anti-botulinum phage (Table 1). We have presumed that the increase in the phage titer in the cell lysate compared to the last stripping wash is due to endocytosed phage. In fact, some of these phage could have come from the cell surface or intracellular matrix. While this could be true for a fraction of the phage from the cell lysate, the immunofluorescence results indicate that at least some of the phage are endocytosed. One indicator of the relative fraction of endocytosed phage for the different C6.5 molecules is to compare the amount of phage remaining on the cell surface prior to cell lysis (last stripping wash) with the amount recovered after cell lysis. This ratio shows only a minor increase for monovalent C6.5 scFv or C6ML3-9 scFv phagemid (6.8 and 8.4 fold

respectively) compared to anti-botulinum phagemid (5.4) (Table 2). In contrast the ratios for dimeric C6.5 diabody phagemid and multivalent C6.5 scFv phage increase to a greater extent (35 and 39 respectively) compared to anti-botulinum phagemid.

4) Increasing the enrichment ratios of specifically endocytosed phage

The results above indicate that phage antibodies can undergo receptor mediated endocytosis and remain infectious in a cell lysate. Selection of internalized phages from a phage library requires the optimization of the method to increase enrichment of specifically internalized phages over non-internalized phage. Two parameters can be improved: (1) reduction of the recovery of non-specific or non-internalized phage and (2) preservation of the infectivity of internalized phage. To examine these parameters, we studied wild-type C6.5 scFv phagemid. We chose this molecule because it was clearly endocytosed based on confocal microscopy, yet was the molecule undergoing the least degree of specific endocytosis. C6.5 scFv phagemid also represents the most commonly utilized format for display of non-immune phage antibody libraries (single copy pIII in a phagemid vector) and has an affinity (16 nM) more typical of Kd's of scFv from such libraries than the affinity matured C6ML3-9 scFv (10, 23).

4a) Reducing the background of non-internalized phage

To reduce the background of non-specific phage recovery, we studied the effect of trypsinizing the cells prior to TEA lysis. This should remove phage trapped in the extracellular matrix. Trypsinization also dissociates the cells from the cell culture flask, permitting transfer to a new vessel and elimination of any phage bound to the cell culture flask. For these experiments, C6.5 scFv phagemid (5.0×10^8 ampicillin resistant cfu) were mixed with a 1000 fold excess of wild type fd phage (5.0×10^{11} tetracylcine resistant cfu). After incubation of phagemid with SKBR-3 cells for 2 hours at 37°C, cells were washed with PBS and three times with stripping buffer. Cells were then directly lysed with TEA or treated with trypsin, washed twice with PBS and then lysed with TEA. Phagemid in the first stripping wash and the cell lysate were titered by infection of *E. coli* TG1 and plated on ampicillin and tetracycline plates. The titer of fd phage and

C6.5 scFv phagemid recovered from the cell surface was comparable for the two experimental groups (Figure 4). The ratio of fd phage/C6.5 scFv phagemid in the cell surface fractions (160/1 and 250/1) yields a 4 to 6 fold enrichment achieved by specific cell surface binding from the initial 1000 fold ratio. Without trypsinization, the ratio of fd phage /C6.5 scFv phagemid in the cell lysate increases only 6.1 fold; in contrast, the ratio increases 209 fold with trypsinization (figure 4). This results from a 60 fold reduction in non-specific binding with only a minor reduction in the amount of specific phage recovery (figure 4).

4b) Improving the recovery of infectious internalized phage

To increase the recovery of infectious internalized phage, we studied whether prevention of lysosomal acidification through the use of chloroquine would protect endocytosed phages from endosomal degradation (12). SKBR3 cells were incubated with chloroquine and either C6.5 scFv phagemid or anti-botulinum phagemid. Cell lysates were titered at various time points to determine the number of intracellular phagemid. C6.5 scFv phagemid were present at the 20 minute time point and the amount of phagemid was comparable with or without the addition of chloroquine. At later time points, approximately twice as much infectious phagemid was recovered with the use of chloroquine. In contrast, much lower amounts of anti-botulinum phage were present and chloroquine had no effect on the titer, suggesting that the phagemid result from non-specific surface binding rather than non-specific endocytosis into endosomes. Overall, the results indicate that prevention of lysosomal acidification increases the amount of infectious phage recovered for incubations longer than 20 minutes.

5) Recovery of internalized phage at low phage concentrations

Only very large phage antibody libraries containing more than 5.0×10^9 members are capable of generating panels of high affinity antibodies to all antigens (10, 23, 24). Since phage can only be concentrated to approximately 10^{13} cfu/ml, a typical phage preparation from a large library will only contain 10^4 copies of each member. Thus selection of libraries for endocytosis could only work if phage can be recovered when applied to cells at titers as low as 10^4. We therefore determined the recovery of infectious phage from within SKBR3 cells as a function of the

phage titer applied. SKBR3 cells were incubated with C6.5 scFv, C6ML3-9 scFv or C6.5 diabody phagemids or C6.5 scFv phage for 2 hours at 37°C. Cells were washed three times with stripping buffer, trypsinized and washed twice with PBS. Cells were lysed and intracellular phage titered on *E. coli* TG1. Phage recovery increased with increasing phage titer for all phage studied (figure 6). For monovalently displayed antibodies, phagemid could not be recovered from within the cell at input titers less than 3.0×10^5 (C6.5 scFv) to 3.0×10^6 (C6ML3-9 scFv) This threshold decreased for bivalent and multivalent display (3.0×10^4 for C6.5 diabody phagemid and C6.5 scFv phage).

Discussion

We demonstrate for the first time that phage displaying an anti-receptor antibody can be specifically endocytosed by receptor expressing cells and can be recovered from the cytosol in infectious form. The results demonstrate the feasibility of directly selecting internalizing antibodies from large non-immune phage libraries and identify the factors that will lead to successful selections. Phage displaying anti-ErbB2 antibody fragments are specifically endocytosed by ErbB2 expressing SKBR3 cells, can be visualized within the cytosol and can be recovered in an infectious form from within the cell. When monovalent scFv antibody fragments were displayed monovalently in a phagemid system, recovery of internalized phage was only 3.5 to 18 fold above background. Display of bivalent diabody or multivalent display of scFv in a phage vector increased recovery of internalized phage to 30 to 146 fold above background. This result is consistent with our studies of native monomeric C6.5 scFv and dimeric C6.5 diabody as well as studies of other monoclonal anti-ErbB2 antibodies where dimeric IgG but not monomeric Fab dimerize and activate the receptor and undergo endocytosis (7, 8). In fact it is likely that endocytosis of C6.5 and C6ML3-9 scFv phagemids reflect the small percentage of phage displaying two or more scFv (21). The importance of valency in mediating either high avidity binding or receptor crosslinking and subsequent endocytosis is confirmed by the only other report demonstrating specific phage endocytosis. Phage displaying approximately 300 copies of a high affinity Arg-Gly-Asp integrin binding peptide on pVIII were efficiently endocytosed by

mammalian cells (11). Recovery of phage after endocytosis also increases the specificity of cell selections compared to recovery of phage from the cell surface. Thus enrichment ratios for specific vs non-specific surface binding range from 2 to 20 fold. These values are comparable to the approximately 10 fold enrichment reported by others for a single round of cell surface selection (25, 26). In contrast our enrichment ratios for specific vs non-specific endocytosis range from 3.5 to 146 fold.

Based on these results, selection of internalizing antibodies from phage antibody libraries would be most successful with either homodimeric diabodies in a phagemid vector or multivalent scFv using a phage vector. While no such libraries have been published, there are no technical barriers preventing their construction. Multivalent libraries would present the antibody fragment in the form most likely to crosslink receptor and undergo endocytosis. Antibodies from such libraries would need to be bivalent to mediate endocytosis. Alternatively, monomeric receptor ligands can activate receptors and undergo endocytosis, either by causing a conformational change in the receptor favoring the dimeric form or by simultaneously binding two receptors. Monomeric scFv that bound receptor in a similar manner could also be endocytosed. Thus selection of libraries of monovalent scFv in a phagemid vector could result in the selection of ligand mimetics that activate receptors and are endocytosed as monomers. Such scFv could be especially useful for the construction of fusion molecules for the delivery of drugs, toxins or DNA into the cytoplasm. Since antibodies which mediate receptor internalization can cause receptor down regulation and growth inhibition (8, 27-29), selection for endocytosable antibodies may also identify antibodies which directly inhibit or modulate cell growth.

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Figure 1: Internalization of soluble C6.5 scFv and diabody. SKBR3 cells grown on cover slips were incubated with (A) anti-botulinum scFv (B) C6.5 scFv or (C) C6.5 diabody for 2 hours at 37°C. Coverslips were washed with PBS and stripping buffer, cells were fixed and permeabilized and intracellular antibodies were detected by confocal microscopy using the anti-myc antibody 9E10, anti-mouse biotinylated antibody and streptavidin Texas-Red.

Figure 2: Antibody phage display: Cartoon of phage displaying (A) a single scFv (B) a single diabody or (C) multiple scFv. scFv = single chain Fv antibody fragment; V_H = Ig heavy chain variable domain; V_L = Ig light chain variable domain; pIII = phage minor coat protein pIII; Ag = antigen bound by scFv.

Figure 3: Internalization of C6.5 phage derivatives. SKBR3 cells grown on coverslips were incubated with 5.0×10^{11} cfu/ml (A) anti-botulinum phagemid (B) scFv C6.5 phagemid (C) C6ML3-9 phagemid (D) C6.5 diabody phagemid or (E) C6.5 phage for 2 hours at 37°C. The cells were treated as described in figure 1 and intracellular phage were detected with a fluorescent microscope using biotinylated anti-M13 antiserum and Texas-Red streptavidin.

Figure 4: Effect of trypsinization on the enrichment of antigen specific phage. A mixture of fd phage (5.0×10^{11} cfu) and C6.5 scFv phagemid (5.0×10^{8} fu) was incubated with SKBR3 cells for 2 hours at 37°C. Washes were performed either as described in Table 1 (-) or cells were trypsinized prior to cell lysis (+). Phage present in the first stripping buffer wash (cell surface phage) and the cell lysate (intracellular phage) were titered in the presence of ampicillin (C6.5 phagemid) or tetracycline (fd phage).

Figure 5: Effect of incubation time and chloroquine on the recovery of antigen specific phage. SKBR3 cells were incubated in the presence (■, ●) or absence (□, ○) of chloroquine (50 μM) for 2 hours prior to the addition of anti-botulinum phagemid (□, ■) or C6.5

scFv phagemid (○, ●) (1.5×10^9 cfu/ml). Cell samples were taken at 0 minutes, 20 minutes, 1 hour or 3 hours after phage addition, washed as described in figure 4 including the trypsinization step and intracellular phages titered.

Figure 6: Effect of phage concentration on the recovery of intracellular phage. Various concentrations of C6.5 scFv phagemid, C6ML3-9 scFv phagemid, C6.5 diabody phagemid or C6.5 scFv phage (input phage titer) were incubated with subconfluent SKBR3 cells grown in 6-well plates for 2 hours at 37°C. Cells were treated as described in figure 4 including the trypsinization step and intracellular phage were titered (output phage titer).

Phage Antibody	*Cell Surface Phage Titer (x 10^{-5})*			*Intracellular Phage Titer (x 10^{-5})*
	1st Wash	*2nd Wash*	*3rd Wash*	
Anti-botulinum phagemid	280	36	2.8	15
C6.5 scFv phagemid	600	96	7.6	52
C6ML3-9 scFv phagemid	2500	140	32	270
C6.5 diabody phagemid	1800	120	13	450
C6.5 scFv phage	2300	620	56	2200

Table 1: Titration of membrane bound and intracellular phage. 3.0 x 10^{11} cfu of monovalent C6.5 scFv phagemid, 16 fold higher affinity monovalent C6ML3-9 scFv phagemid, bivalent C6.5 diabody phagemid or multivalent C6.5 fd phage were incubated with sub confluent SKBR3 cells for 2 hours at 37°C. Cells were washed 6 times with PBS, 3 times with stripping buffer and then lysed to recover intracellular phage. The various fractions were neutralized and the phage titered. The total number of cfu of each fraction is reported. Non specific anti-botulinum phagemid were used to determine non specific recovery.

Phage Antibody	*Anti-ErbB2 /Anti-Botulinum Phage Titer Ratio**			*Intracellular/ Cell Surface Phage Ratio***
	Cell surface (1st Wash)	*Cell surface (3rd Wash)*	*Intracellular*	
C6.5 scFv phagemid	2.14	2.7	3.5	6.8
C6ML3-9 scFv phagemid	8.9	11.4	18	8.4
C6.5 diabody phagemid	6.4	4.6	30	35
C6.5 scFv phage	8.2	20	146	39

Table 2: Specific enrichment of anti-ErbB2 phage compared to anti-botulinum phage. *The titers of anti-ErbB2 phage are divided by the titers of the anti-botulinum phage (Table 1) to derive an enrichment ratio for specific vs nonspecific binding or internalization. **The titer of intracellular phage is divided by the titer of cell surface bound phage (Table 1) to derive the ratio of internalized phage vs surface bound phage.

FIGURE 1

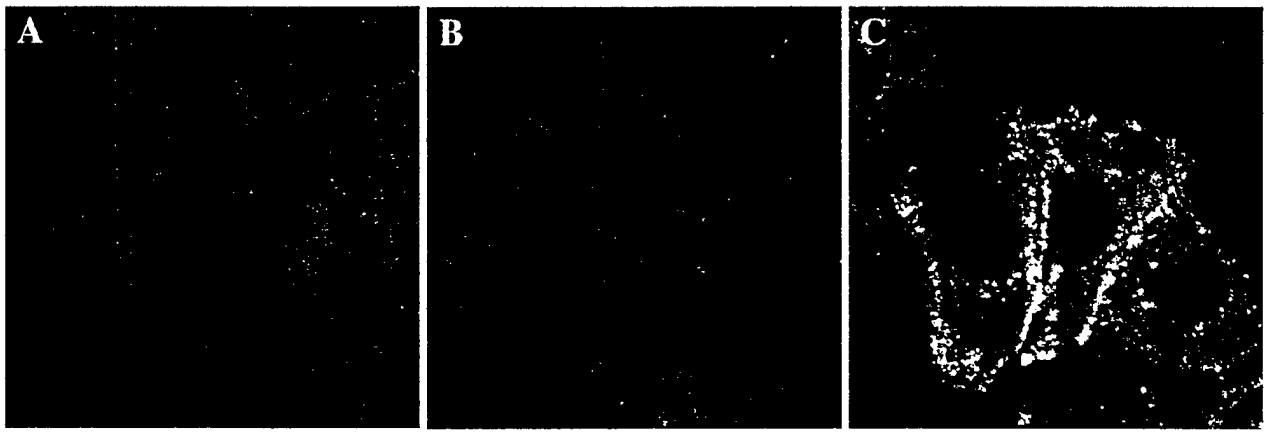
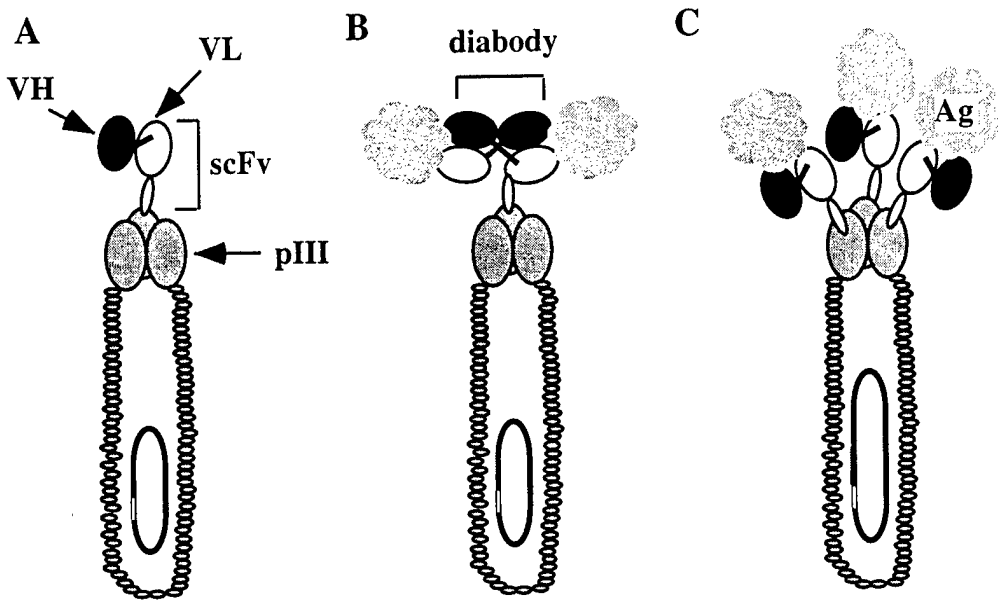


FIGURE 2



scFv phagemid

diabody phagemid

scFv phage

FIGURE 3

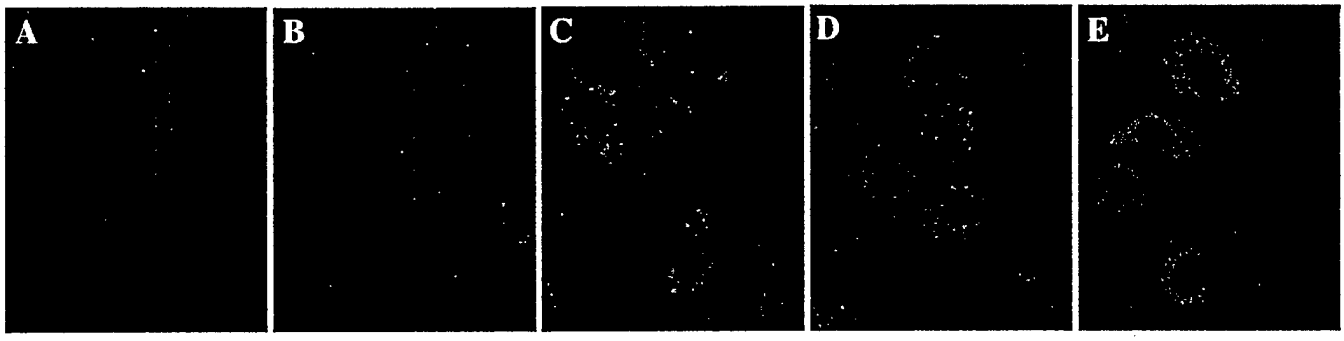


FIGURE 4

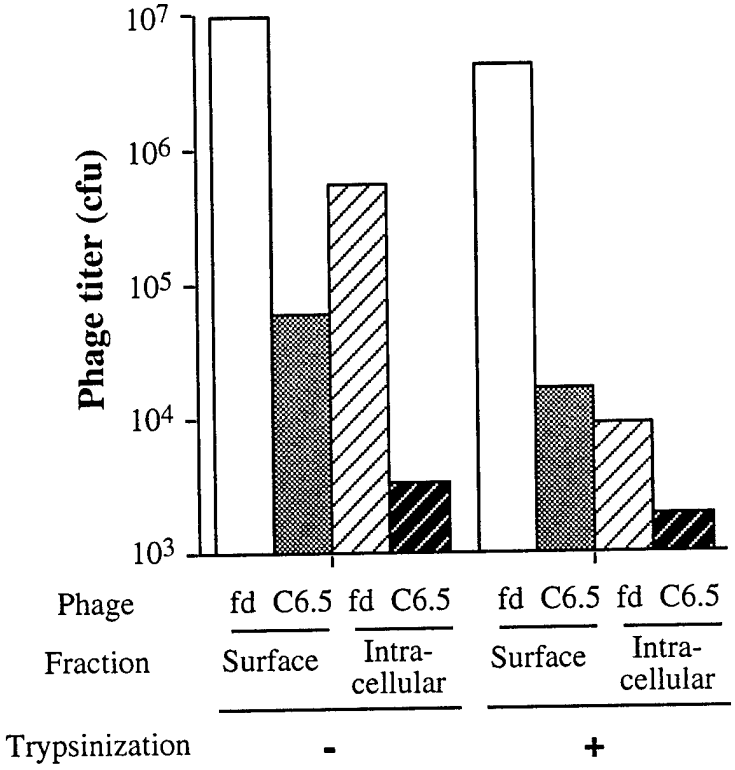

Phage titer (cfu)
10^7
10^6
10^5
10^4
10^3
Phage
fd C6.5 fd C6.5 fd C6.5 fd C6.5
Fraction
Surface
Intra-cellular
Surface
Intra-cellular
Trypsinization
-
+

FIGURE 5

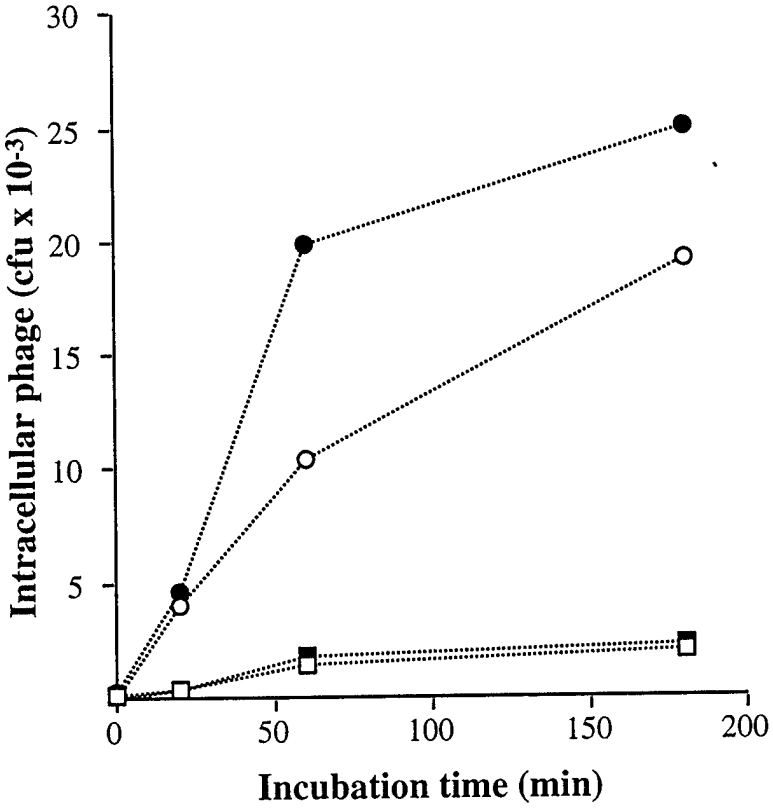
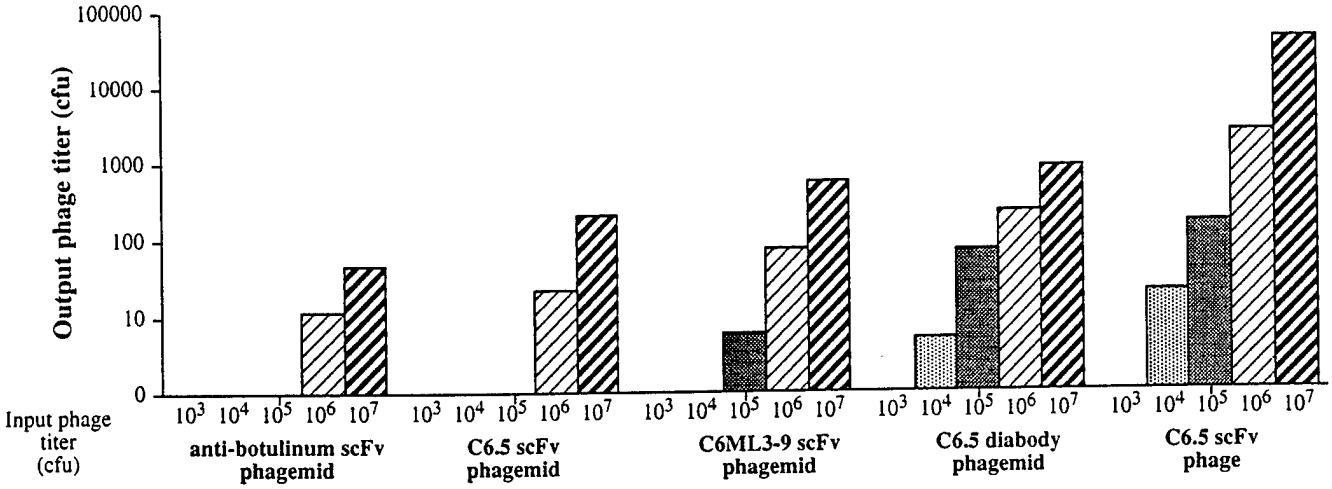

30
25
20
15
10
5
Intracellular phage (cfu x 10-3)
0
50
100
150
200
Incubation time (min)

FIGURE 6


Output phage titer (cfu)
100000
10000
1000
100
10
0
Input phage titer (cfu)
10^3 10^4 10^5 10^6 10^7
anti-botulinum scFv phagemid
C6.5 scFv phagemid
C6ML3-9 scFv phagemid
C6.5 diabody phagemid
C6.5 scFv phage