Corrections

Commentary. In the article “Lignification of plant cell walls: Impact of genetic manipulation” by Hans-Joachim G. Jung and Weiting Ni, which appeared in number 22, October 27, 1998, of Proc. Natl. Acad. Sci. USA (95, 12742–12743), the authors request that the following corrections be noted. It was accidentally stated that the studies by Kajita et al. (1) and Lee et al. (2) dealt with cinnamoyl-CoA reductase modified plants when in fact they concerned 4-coumarate:coenzyme A ligase (4CL) transgenic plants. Lignin concentration was reduced by down-regulation of 4CL activity in both studies (1, 2). In a subsequent article, Kajita et al. (3) reported a negligible decrease in lignin concentration and a decreased syringyl-to-guaiacyl ratio for lignin composition of a sense-suppressed 4CL transgenic tobacco line. Kajita et al. (1) rather than Kajita et al. (3) was inadvertently cited when this later report was contrasted with the large decreases in lignin concentration and an increased syringyl-to-guaiacyl lignin ratio for anti-sense suppressed 4CL Arabidopsis transgenics (2). The authors apologize for the confusion these errors have created for readers of their Commentary and to the authors of the cited work for misrepresenting their research.


Biochemistry. In the article “Requirement of G M2 ganglioside activator for phospholipase D activation” by Shun-ichi Nakamura, Toshihiro Akisue, Hitoshi Jinnai, Tomohiro Hitomi, Sukumar Sakar, Noriko Miwa, Taro Okada, Kimihisa Yoshida, Shun’ichi Kuroda, Ushio Kikkawa, and Yasutomii Nishizuka, which appeared in number 21, October 13, 1998, of Proc. Natl. Acad. Sci. USA, the authors request that the following corrections be noted. It was accidentally stated that the study by Kajita et al. (1), Kajita et al. (2), and Lee et al. (3) (4CL) transgenic plants. Lignin concentration was reduced by down-regulation of 4CL activity in both studies (1, 2). In a subsequent article, Kajita et al. (3) reported a negligible decrease in lignin concentration and a decreased syringyl-to-guaiacyl ratio for lignin composition of a sense-suppressed 4CL transgenic tobacco line. Kajita et al. (1) rather than Kajita et al. (3) was inadvertently cited when this later report was contrasted with the large decreases in lignin concentration and an increased syringyl-to-guaiacyl lignin ratio for anti-sense suppressed 4CL Arabidopsis transgenics (2). The authors apologize for the confusion these errors have created for readers of their Commentary and to the authors of the cited work for misrepresenting their research.


Cell Biology. In the article “Impairing follicle-stimulating hormone (FSH) signaling in vivo: Targeted disruption of the FSH receptor leads to aberrant gametogenesis and hormonal imbalance” by Andree Dierich, M. Ram Sairam, Lucia Monaco, Gian Maria Fimia, Anne Gansmuller, Marianne LeMeur and Paolo Sassone-Corsi, which appeared in number 23, November 10, 1998, of Proc. Natl. Acad. Sci. USA (95, 13612–13617), the authors request that the following correction be noted: In Fig. 2 appearing on page 13614, the genotype identification for testicular histology in panels C and D were shown reversed. The correct identification is −/+ for panel C and +/+ for panel D. The fifth sentence of the figure legend should read as follows: “Histological sections at lower (E) and higher (D) magnification of the seminiferous tubuli from a wild-type and mutant (F and C) mouse.”

![Fig. 3](image_url) Fig. 3. Enhancement by PLD activator of enzymatic conversion of G M2 to GM3 ganglioside catalyzed by β-hexosaminidase A. Purified PLD activator was loaded on a Superdex 200 column (Fig. 1). Each fraction was assayed for the ability to stimulate enzymatic conversion of GM2 to GM3 ganglioside catalyzed by β-hexosaminidase A. PLD activation also is plotted in the same figure. ●, PLD activity; ○, GM3 formation.

![Fig. 4](image_url) Fig. 4. Stimulation of PLD by G M2 ganglioside activator or heat-stable PLD activator. (A) Stimulation of PLD by various amounts of purified GM2 ganglioside activator or by heat-stable PLD activator. ● and ○, with GM2 ganglioside activator; ■ and ◆, with heat-stable PLD activator. ● and ■, with 200 nM ARF; ○ and ◆, without ARF. (B) Time course of PLD reaction. ●, with 56 nM GM2 ganglioside activator and 200 nM ARF; ■, with 56 nM heat-stable PLD activator and 200 nM ARF; ◆, with 200 nM ARF alone.

![Corrections](image_url)
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 × 10⁹ 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 (VH) and light (VL) 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 immunotoxins (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 VH and VL 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 × 10⁹ 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 Kₘ for the ErbB2 protein ranging between 220 pM and 4 nM.

METHODS

Construction of the VH Library. Total RNA was prepared from three different samples of human spleen cells and two...
different samples of human peripheral blood lymphocytes, cDNA was synthesized from total RNA primed with the HulMFOR primer (12). VH gene repertoires were amplified from the cDNA by using Vent DNA polymerase (New England Biolabs) in combination with the HulMFOR primer and an equimolar mixture of HuVHBACK primers (12). PCR products were agarose gel-purified and reamplified to append NcoI and NotI restriction sites by using Tth DNA polymerase (Epicentre Technologies, Madison, WI) and an equimolar mixture of the HuVHBACKSfi primers (that contain an NcoI site for cloning) and the HuCMForNot primer (5’-GAGTC-ATTCTGCATCTGGGCGCTGGAAGAGGCACGTTC-TTTCCTT-3’) (12). The PCR products were cut with restriction enzymes NcoI and NotI and agarose gel-purified. The resulting DNA fragments were ligated into the plasmid pCITE3A (Novagen) cut with restriction enzymes NcoI and NotI and the ligated DNA was electroporated into the E. coli strain TG1. A library of VH 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-VH. Cloning efficiency and library diversity was determined by PCR screening (12, 16). The pCITE3A plasmid was used to create the VH gene repertoire because of the presence of unique sequences for PCR amplification that surround the NcoI and NotI cloning sites. These sequences allow the specific amplification of the VH genes for scFv assembly. This strategy is advantageous for amplification of the VH genes and also the subsequent amplification of scFv genes assembled from the VH genes. Although we chose the pCITE3A plasmid for production of our VH 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 VH gene repertoire was PCR-amplified from the pCITE-VH library by using 300 ng of library plasmid DNA as a template, Vent DNA polymerase, the CITE3 primer (5’-GATCTGATCTGGGCGCTGGAAGAGGCACGTTC-TTTCCTT-3’), and an equimolar mixture of HuJH primers (12). The VL genes for scFv assembly were obtained from a previously constructed scFv phage antibody library (12). The VL gene repertoire, including DNA encoding the scFv peptide linker (G4S)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 RHuJH primers (12). The amplified VH and VL genes were agarose gel-purified and spliced together with overlap extension PCR to create a scFv gene repertoire (11). To accurately join VH and VL 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 VH and VL DNA fragments for scFv assembly. For all subsequent PCR steps of library construction, Tth DNA polymerase was found to be the optimal enzyme. The VH and VL gene repertoires were spliced together in 100-μl PCRs containing 100 ng of the VH and VL 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 NcoI and NotI agarose gel-purified, and ligated into the plasmid pHEN-1 (17) cut with NcoI and NotI. 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. 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 cytokeratin b5 was provided by by Lucy Waskell (Univ. of California, San Francisco), 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 scFvs were affinity-selected by using proteins absorbed to Immunotubes (Nunc) (12). For selections with malse 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 biotylated Erb-B2 ECD and

![FIG. 1. Schematic outline of the approach used for library construction. A library of VH and genes was generated from rearranged human V-genes and cloned into the plasmid pCITE3A. The VH genes used for scFv assembly were derived from a previously constructed scFv library contained in the plasmid pHEN1 (12). The vector containing the VH repertoire also contained the scFv linker DNA 5′ to the VH genes. Primers for reamplification of the V-gene repertoires were derived from sequences several hundred bp 5′ (the VH genes) or 3′ (the VL 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) VH and linker-VL gene repertoires were generated by PCR from the plasmid DNA of the separate libraries. The VH genes were amplified by using a plasmid specific primer (GA) and an equimolar mixture of HuJH primers (GA). The linker DNA and VL genes were amplified by using a plasmid specific primer (GA) and an equimolar mixture of RHuJH primers (GA). The RHuJH primers are complementary to the HuJH primers. (B) The VH and linker DNA-VL gene repertoires were PCR assembled into a scFv gene repertoire. (C) The assembled scFv gene repertoire was cut with the restriction enzymes NcoI and NotI and cloned into the plasmid pHEN1 (17) for phage display.](image-url)
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 L292 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*<sub>H</sub> and *V*<sub>L</sub> 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*<sub>d</sub>) were calculated from the association (*k*<sub>a</sub>) and dissociation (*k*<sub>off</sub>) 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<sup>6</sup> inclusion forming units/ml 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*<sub>H</sub> and *V*<sub>L</sub> gene repertoires contained in separate plasmid vectors. A library of *V*<sub>H</sub> genes, containing 2.3 × 10<sup>8</sup> members, was specifically created for generating an additional scFv repertoire. The *V*<sub>L</sub> genes for scFv assembly were derived from an existing scFv repertoire containing 3.0 × 10<sup>9</sup> members (Fig. 1A). 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*<sub>H</sub> and *V*<sub>L</sub> 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 (G4S)<sub>3</sub> linker located at the 5′ end of the *V*<sub>L</sub> library (Fig. 1B). Using *V*<sub>L</sub> 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*<sub>H</sub> and *V*<sub>L</sub> gene repertoires and linker DNA (12). Third, the *V*<sub>H</sub> and *V*<sub>L</sub> gene repertoires and the scFv genes assembled from these repertoires were amplified with primers that annealed to sequences approximately 200 bp 5′ of the *V*<sub>H</sub> genes and to sequences approximately 200 bp 3′ of the *V*<sub>L</sub> genes. This strategy generated long sequence extensions at the ends of the individual *V*<sub>H</sub>, *V*<sub>L</sub>, and scFv 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. 1C).

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<sup>9</sup> 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*<sub>H</sub> CDR3 length of unselected and antigen-specific scFv. The *V*<sub>H</sub> and *V*<sub>L</sub> 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*<sub>H</sub>, *V*<sub>L</sub>, and *V*<sub>λ</sub> genes are listed on the ordinate, with the *V*<sub>H</sub>, *V*<sub>κ</sub>, or *V*<sub>λ</sub> germ-line gene family indicated below. Only V-genes in unselected or selected clones are listed.
scFv specificity is shown in Fig. 4, where a homology between the toxins (Fig. 3). Another example of the four different types of BoNT, despite 32–59% sequence example, serotype specific scFv were isolated against each of clones would yield additional antibodies.

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 proteins antigen, with the number of scFv ranging from 3 to 15 

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

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 \( \text{H} \) complementarity determining region (CDR) 3 length of between 5 and 18 residues (Fig. 2). There was, however, bias in V \( \text{H} \)-gene usage, with both over-representation of specific V \( \text{H} \)-gene families (\( V_{\text{H}3}, V_{\text{L}1}, \) and \( V_{\text{L}3} \)) and V-genes (DP-47, DPL 16) (Fig. 2). This bias partially reflects differential V-\( \text{H} \)-gene usage observed in the human B-cell repertoire (30–33) and also in the unselected library (for example DP-47 and DPL-16). Differential V-\( \text{H} \)-gene usage also may reflect expression biases of the 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 from 26 different germ-line genes. \( V_{\text{L}} \) genes were derived from three of the six \( V_{\text{H}} \) gene families (nos. 1, 3, and 4) and 11 different \( V_{\text{L}} \) germ-line genes, from three of the nine \( V_{\text{H}} \) gene families (nos. 1–3), and nine different \( V_{\text{L}} \) germ-line genes. Despite the diversity, there was a bias seen in the \( V_{\text{H}} \)-gene usage. \( V_{\text{H}1} \) genes largely were derived from the \( V_{\text{H}3} \) family, particularly DP46 and DP47. \( V_{\text{L}} \) genes most frequently were derived from the \( V_{\text{L}1} \) family while \( V_{\text{L}} \) genes most frequently were derived from the \( V_{\text{L}3} \) family, especially DPL-16. This bias partially reflects the greater frequency of certain V-genes in the B-cell repertoire (30–33) and also in the unselected library (for example DP-47 and DPL-16). Differential V-\( \text{H} \)-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). \( V_{\text{H}} \) 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 \( V_{\text{H}} \) 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
Table 2. Affinities and binding kinetics of anti-BoNT A C-fragment and anti-Erb-B2 scFv

<table>
<thead>
<tr>
<th>Specification and clone</th>
<th>$K_a$ ($\times 10^{-9}$ M)</th>
<th>$k_{on}$ ($\times 10^9$ M$^{-1}$s$^{-1}$)</th>
<th>$k_{off}$ ($\times 10^{-3}$ s$^{-1}$)</th>
</tr>
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<tbody>
<tr>
<td>ErbB-2 B7A</td>
<td>0.22</td>
<td>4.42</td>
<td>0.1</td>
</tr>
<tr>
<td>ErbB-2 G11D</td>
<td>0.48</td>
<td>2.19</td>
<td>0.11</td>
</tr>
<tr>
<td>ErbB-2 A11A</td>
<td>0.49</td>
<td>3.69</td>
<td>0.18</td>
</tr>
<tr>
<td>ErbB-2 F5A</td>
<td>4.03</td>
<td>1.62</td>
<td>0.65</td>
</tr>
<tr>
<td>BoNT-A 2A9</td>
<td>26.1</td>
<td>0.25</td>
<td>0.66</td>
</tr>
<tr>
<td>BoNT-A 2H6</td>
<td>38.6</td>
<td>2.2</td>
<td>8.5</td>
</tr>
<tr>
<td>BoNT-A 2F6</td>
<td>66.0</td>
<td>4.7</td>
<td>30.9</td>
</tr>
<tr>
<td>BoNT-A 2B6</td>
<td>71.5</td>
<td>1.1</td>
<td>7.8</td>
</tr>
</tbody>
</table>

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

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

<table>
<thead>
<tr>
<th>Library</th>
<th>Library size and type*</th>
<th>Number of protein antigens studied</th>
<th>Average number of antibodies per protein antigen</th>
<th>Number of affinities measured</th>
<th>Range of affinities for protein antigens $K_a$ ($\times 10^{-9}$ M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marks et al. (12)</td>
<td>$3.0 \times 10^7$ (scFv, N)</td>
<td>2</td>
<td>2.5</td>
<td>1</td>
<td>100–2000</td>
</tr>
<tr>
<td>Nissim et al. (13)</td>
<td>$1.0 \times 10^8$ (scFv, SS)</td>
<td>15</td>
<td>2.6</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>deKruif et al. (42)</td>
<td>$3.6 \times 10^8$ (scFv, SS)</td>
<td>12</td>
<td>1.9</td>
<td>3</td>
<td>100–2,500</td>
</tr>
<tr>
<td>Griffiths et al. (14)</td>
<td>$6.5 \times 10^9$ (Fab, SS)</td>
<td>30</td>
<td>4.8</td>
<td>3</td>
<td>7.0–58</td>
</tr>
<tr>
<td>Vaughan et al. (15)</td>
<td>$1.4 \times 10^{10}$ (scFv, N)</td>
<td>3</td>
<td>7.0</td>
<td>3</td>
<td>4.2–8.0</td>
</tr>
<tr>
<td>Sheets et al. (this work)</td>
<td>$6.7 \times 10^7$ (scFv, N)</td>
<td>14</td>
<td>8.7</td>
<td>8</td>
<td>0.22–71.5</td>
</tr>
</tbody>
</table>

*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 VH CDR3. ND, not determined.
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