Synthesis of Ligand-Specific Phage-Display ScFv against the Herbicide Picloram by Direct Cloning from Hyperimmunized Mouse

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Immunoglobulin genes were directly isolated from the splenocytes of a BALB/C mouse hyperimmunized with the auxinic herbicide picloram conjugated to bovine serum albumin. Variable light and heavy domain DNA were joined to produce single-chain Fv (scFv) DNA, which was cloned into phage vector fd-tet-GIIID to display multiple copies of scFv on the filamentous phage minor coat protein gIIIp. The phage-display scFv library (10^4 clones) was selected against picloram conjugated to ovalbumin. After five rounds of panning, individual clones were analyzed. ScFv with different affinities to picloram (IC_{50} values ranging from 20 ppb to 10 ppm) were detected in the final enriched pool. The increased avidity of the phage vector enhanced the selection (i.e., panning) of multiple picloram-specific recombinant antibodies. Stringent selection was required to isolate the clones with the highest affinity. Nucleotide sequence analysis of six isolated clones revealed that all of the V_L belonged to the *Vκ9A* family joined to *Jκ2* segments. All of the V_H belonged to the *V_H7183* family and joined to two different J segments (i.e., *J_H2* or *J_H4*). Different from the immune response to large molecular weight molecules (MW > 10000 Da), which requires both VDJ segment rearrangement and somatic hypermutations, production of high-affinity antibodies to picloram, a small ligand having a formula weight of 241.5 Da, predominantly requires somatic hypermutations.

Keywords: *ELISA; herbicides; hyperimmunized mouse; library; panning; PCR; phage display; picloram; scFv; spleen*

INTRODUCTION

The versatility and flexibility of antibody (Ab) technology can be used in agriculture in numerous ways. Antibodies can be used in immunoassays to monitor agrochemicals in the environment for registration and stewardship purposes (*1–3*), in affinity chromatography for extraction and purification of ligands of interest (*4*), and to screen potential lead chemistry for development of new drugs and pesticides (*5–7*). Production of recombinant Abs using phage-display technology has many advantages over conventional polyclonal and monoclonal Ab production (*2, 8, 9*). Through phage-display technology and Ab engineering, the use of animals for Ab production can be minimized and eventually eliminated. More importantly, the coselection of desired Abs and the genes encoding them allows for many novel applications of Abs. Mutagenesis and affinity selection of immunoglobulin genes can create Abs with different affinities and specificities (*9*). Furthermore, following the selection through phage-display technology, the selected Ab genes can be expressed in various expression systems such as plants (*10, 11*). Expression systems may potentially reduce the cost of Ab production and widen the scope of Ab applications. For example, Abs produced in plants may be used for in vivo immunomodulation, protection against pathogens, and bioremediation and to impart resistance to phytotoxic pesticides (*10–12*).

Immunoglobulin genes have been successfully cloned from hybridoma cell lines that produce Abs specific to antigens ranging from human self-antigens (*13*) and pathogens (*14, 15*) to small chemical molecules (*2, 5*). Obtaining and maintaining a stable hybridoma cell line, however, is labor intensive and in some cases problematic (*9*). Theoretically, each hybridoma cell line produces only a single type of functional Ab; therefore, only one set of immunoglobulin genes is available. However, depending on the myeloma cell line used in the fusion process, rearranged but nonfunctional heavy and light chain genes in the hybridoma may be present and preferentially amplified and cloned (*16*). To select for functional immunoglobulin genes from hybridoma cell lines, intensive screening of cloned genes through panning of phage-display Abs is necessary (*3, 5*).

Immunotechnology can be widely applied in agriculture and the food industry, especially for the purpose of agrochemical detection and monitoring using immunoassays. An effective and reliable method to produce antibodies with high specificity and affinity would be desirable. We report here the de novo selection of DNA encoding single-chain Fv (scFv) specific to the auxinic herbicide picloram (4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid) directly from splenocytes of a hyperimmunized mouse using phage-display technology. The cloning and panning strategies are described, and the recovered scFv are characterized.

MATERIALS AND METHODS

Immunization. Five 8-week-old BALB/C mice were immunized with picloram conjugated to bovine serum albumin (Pic−BSA) according to methods described by Fleeker (*17*). For the primary immunization, the immunogen was diluted in sterile phosphate-buffered saline (PBS; 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, and 0.24 g of KH_2PO_4 per liter of

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Picloram ScFv from Mouse Splenocytes

J. Agric. Food Chem., Vol. 49, No. 8, 2001 **3629**

water, pH adjusted to 7.4 by 5 M NaOH) and mixed with an equal volume of Freund's incomplete adjuvant (Sigma-Aldrich Chemical Co., St. Louis, MO). Each mouse was injected intraperitoneally with 70 μg of Pic−BSA in a total volume of 250 μL and allowed to rest for 3 weeks. Secondary immunizations were performed at 2-week intervals with the same preparation of immunogen without the adjuvant. Blood samples were collected a week after each boost to monitor the immune response against picloram using a competitive indirect enzyme-linked immunosorbent assay (CI-ELISA) (*2*). After the 12th immunization, the mouse whose serum possessed the lowest IC_{50} value was given a final injection of 140 μg of immunogen in PBS. Three days later, the mouse was sacrificed by exposure to 99% carbon dioxide.

Preparation of Spleen Cells. Spleens were excised and splenocytes released from the sac by perfusion in RPMI medium (Life Technologies, Gaithersburg, MD). The cell suspension was centrifuged at 500*g* for 10 min at 4 °C. The cell pellet contained both red blood cells and splenocytes. The red blood cells were lysed by resuspending the pellet in 5 mL of prechilled (4 °C) sterile PBS containing 0.17 M NH_4Cl followed by incubation on ice for 10 min. Ice-cold RPMI medium (10 mL) was added to the suspension, which was centrifuged a second time to collect the splenocytes. Cell density was estimated by direct cell count with magnification at 400× after staining with Trypan Blue (Sigma-Aldrich Chemical Co.).

Total RNA Extraction. Total RNA was isolated from 10^7 splenocytes using TRIzol reagent (Life Technologies) according to the manufacturer's instructions. The purity and concentration of the RNA were determined by UV spectrophotometry and electrophoresis (*18*). First-strand complementary DNA (cDNA) was synthesized using the First Strand cDNA synthesis kit (Amersham-Pharmacia Biotech, Piscataway, NJ). Total RNA (5 μg in 20 μL) was mixed with 11 μL of bulk First-Strand reaction mix, 1 μL (0.2 μg/μL) of oligo-dT primer, and 1 μL of 200 mM dithiothreitol. The mixture was incubated at 37 °C for 1 h.

ScFv DNA Construction. Five microliters of the first-strand cDNA product was used as a template for the amplification of the variable heavy (V_H) and light (V_L) domains of the immunoglobulin genes by Polymerase Chain Reaction (PCR) following the protocol of Krebber et al. (*19*). The primers used for primary V_H and V_L gene amplification (Table 1) were modified by adding *Apa*LI and *Not*I sites to the 5′ ends to facilitate directional cloning of the products into the phage vector fd-tet-GIIID, developed by MacKenzie and To (*20*). PCR products were separated and purified by agarose gel electrophoresis.

A second round of PCR was used to introduce the linker sequence to both V_L and V_H. Two oligonucleotides encoding the linker sequence $(Gly_4Ser)_3$ were used as primers. Primer LinkerT (Table 2) encoding the sense strand of the linker sequence annealed to the 5′ end of the V_H. Primer LinkerB (Table 2) encoding the antisense strand of the linker sequence annealed to the 3′ end of the V_L. Ten microliters (20 ng) of each of the V_L and V_H products was mixed with 15 pmol each of the LinkerB and LinkerT primers. PCR was performed in a 50-μL volume containing 10 mmol of dNTP and 5 units of *Taq* polymerase. DNA was denatured at 94 °C for 3 min, followed by a 25-cycle amplification of 1 min at 94 °C, 1.5 min at 60 °C, and 2 min at 72 °C.

At the beginning of the third round of PCR, V_L and V_H were self-primed through the complementarity of the linker sequence and spliced together. Two oligonucleotides, pApaL1 and pNot1 (Table 2), annealed to the 5′ end of V_L and 3′ end of V_H, respectively, and amplified the linked variable fragments to form the scFv DNA. PCR was performed with 5 μL of the product from the previous PCR as template. Using the same reaction conditions as in the second PCR, a 750-bp product was amplified, isolated, and purified by agarose gel electrophoresis.

Construction of Phage-Display ScFv Library. The phage vector fd-tet-GIIID displayed five copies of scFv covalently linked to the minor coat protein gIIIp (*20*). The

Table 1. Universal Primers for Primary PCR Amplification of V_H and V_L of Mouse Antibodies (Modified from Reference *19*)[a]

Heavy Forward Mix (HF)

		*Not*I	
HF1	5′ GAG TCA TTC T	**GC GGC CGC**	CGA GGA AAC GGT GAC CGT GGT 3′
HF2	5′ GAG TCA TTC T	**GC GGC CGC**	CGA GGA GAC TGT GAG AGT GGT 3′
HF3	5′ GAG TCA TTC T	**GC GGC CGC**	CGC AGA GAC AGT GAC CAG AGT 3′
HF4	5′ GAG TCA TTC T	**GC GGC CGC**	CGA GGA GAC GGT GAC TGA GGT 3′

Heavy Reverse Mix (HB)

		G G G G S	
HB1	5′	<u>GGC GGC GGT GGA TCC</u>	GAK GTR MAG CTT CAG GAG TC 3′
HB2	5′	<u>GGC GGC GGT GGA TCC</u>	GAG GTB CAG CTB CAG CAG TC 3′
HB3	5′	<u>GGC GGC GGT GGA TCC</u>	CAG GTG CAG CTG AAG SAS TC 3′
HB4	5′	<u>GGC GGC GGT GGA TCC</u>	GAG GTC CAR CTG CAA CAR TC 3′
HB5	5′	<u>GGC GGC GGT GGA TCC</u>	GAG GTY CAG CTB CAG CAR TC 3′
HB6	5′	<u>GGC GGC GGT GGA TCC</u>	CAG GTY CAR CTG CAG CAG TC 3′
HB7	5′	<u>GGC GGC GGT GGA TCC</u>	CAG GTC CAC GTG AAG CAG TC 3′
HB8	5′	<u>GGC GGC GGT GGA TCC</u>	GAG GTG AAS STG GTG GAA TC 3′
HB9	5′	<u>GGC GGC GGT GGA TCC</u>	GAV GTG AWG YTG GTG GAG TC 3′
HB10	5′	<u>GGC GGC GGT GGA TCC</u>	GAG GTG CAG SKG GTG GAG TC 3′
HB11	5′	<u>GGC GGC GGT GGA TCC</u>	GAK GTG CAM CTG GTG GAG TC 3′
HB12	5′	<u>GGC GGC GGT GGA TCC</u>	GAG GTG AAG CTG ATG GAR TC 3′
HB13	5′	<u>GGC GGC GGT GGA TCC</u>	GAG GTG CAR CTT GTT GAG TC 3′
HB14	5′	<u>GGC GGC GGT GGA TCC</u>	GAR GTR AAG CTT CTC GAG TC 3′
HB15	5′	<u>GGC GGC GGT GGA TCC</u>	GAA GTG AAR STT GAG GAG TC 3′
HB16	5′	<u>GGC GGC GGT GGA TCC</u>	CAG GTT ACT CTR AAA GWG TST G 3′
HB17	5′	<u>GGC GGC GGT GGA TCC</u>	CAG GTC CAA CTV CAG CAR CC 3′
HB18	5′	<u>GGC GGC GGT GGA TCC</u>	GAT GTG AAC TTG GAA GTG TC 3′
HB19	5′	<u>GGC GGC GGT GGA TCC</u>	GAG GTG AAG GTC ATC GAG TC 3′

Light Forward Mix (LF)

		S G G G G	
LF1/2	5′	<u>AGA GCC GCC TCC ACC</u>	ACG TTT KAT TTC CAG CTT GG 3′
LF4	5′	<u>AGA GCC GCC TCC ACC</u>	ACG TTT TAT TTC CAA CTT TG 3′
LF5	5′	<u>AGA GCC GCC TCC ACC</u>	ACG TTT CAG CTC CAG CTT GG 3′
LFλ	5′	<u>AGA GCC GCC TCC ACC</u>	ACC TAG GAC AGT CAG TTT GG 3′

Light Reverse Mix (LB)

		*Apa*LI	
LB1	5′ CCG CCG CCG C	**GT GCA C**TC	GAY ATC CAG CTG ACT CAG CC 3′
LB2	5′ CCG CCG CCG C	**GT GCA C**TC	GAY ATT GTT CTC WCC CAG TC 3′
LB3	5′ CCG CCG CCG C	**GT GCA C**TC	GAY ATT GTG MTM ACT CAG TC 3′
LB4	5′ CCG CCG CCG C	**GT GCA C**TC	GAY ATT GTG YTR ACA CAG TC 3′
LB5	5′ CCG CCG CCG C	**GT GCA C**TC	GAY ATT GTR ATG ACM CAG TC 3′
LB6	5′ CCG CCG CCG C	**GT GCA C**TC	GAY ATT MAG ATR AMC CAG TC 3′
LB7	5′ CCG CCG CCG C	**GT GCA C**TC	GAY ATT CAG ATG AYD CAG TC 3′
LB8	5′ CCG CCG CCG C	**GT GCA C**TC	GAY ATY CAG ATG ACA CAG AC 3′
LB9	5′ CCG CCG CCG C	**GT GCA C**TC	GAY ATT GTT CTC AWC CAG TC 3′
LB10	5′ CCG CCG CCG C	**GT GCA C**TC	GAY ATT GWG CTS ACC CAA TC 3′
LB11	5′ CCG CCG CCG C	**GT GCA C**TC	GAY ATT STR ATG ACC CAR TC 3′
LB12	5′ CCG CCG CCG C	**GT GCA C**TC	GAY RTT KTG ATG ACC CAR AC 3′
LB13	5′ CCG CCG CCG C	**GT GCA C**TC	GAY ATT GTG ATG ACB CAG KC 3′
LB14	5′ CCG CCG CCG C	**GT GCA C**TC	GAY ATT GTG ATA ACY CAG GA 3′
LB15	5′ CCG CCG CCG C	**GT GCA C**TC	GAY ATT GTG ATG ACC CAG WT 3′
LB16	5′ CCG CCG CCG C	**GT GCA C**TC	GAY ATT GTG ATG ACA CAA CC 3′
LB17	5′ CCG CCG CCG C	**GT GCA C**TC	GAY ATT TTG CTG ACT CAG TC 3′
LBλ	5′ CCG CCG CCG C	**GT GCA C**TC	GAT GCT GTT GTG ACT CAG GAA TC 3′

[a] Restriction site sequences on the primers are italicized. Linker sequences are underlined. (Degeneracy of nucleotides was represented by **B**:C or G or T; **D**:A or G or T; **K**:G or T; **M**:A or C; **R**:A or G; **S**:C or G; **V**:A or C or G; **W**:A or T; **Y**:C or T).

replicative form (RF) of the phage vector was maintained and propagated in *Escherichia coli* JM101 and was isolated as described in Sambrook et al. (*18*). Both the RF and the scFv DNA were digested by restriction enzymes *Apa*LI and *Not*I. ScFv construct (2 μg) was ligated at 14 °C for 18 h to the digested RF (10 μg) using 20 units of ligase in a final volume of 200 μL. The ligated product was electroporated into *E. coli* TG1 and plated on 2xYT (1.6% tryptone, 1% yeast extract, and 1% NaCl, w/v) agar plates containing tetracycline (tet; 12.5 μg/mL) and incubated at 30 °C for 16 h. A library containing 10^4 colonies was obtained. Cells on each plate were collected by resuspending all of the colonies in 2xYT/tet broth. The cell suspension was incubated at 37 °C for 30 min with shaking at 250 rpm. Phage particles secreted into the growth medium were separated from the host cells by centrifugation at 20000*g* for 10 min at 4 °C. Polyethylene glycol 8000 (Sigma-Aldrich Chemical Co.) and NaCl were added to the supernatant to final concentrations of 3 and 4%, respectively. The mixture was incubated at 4 °C for 1 h to precipitate the phage. After centrifugation at 20000*g* for 30 min at 4 °C, the phage pellet was resuspended in PBS and stored at 4 °C. Phage density was determined as colony forming units (CFU) on 2xYT/tet agar plates as described in Sambrook et al. (*18*).

Panning. Picloram was conjugated to ovalbumin (Pic−OVA) as described previously (*2*). Three wells of an F8

3630 *J. Agric. Food Chem.,* Vol. 49, No. 8, 2001

Tout et al.

Table 2. Nucleotide Sequences of Primers for Linking V_L and V_H Genes Using Overlap/Extension Method Adopted from Krebber et al. (19)[a]

		G	G	G	G	S	G	G	G	G	S	G	G	G	G	S
LinkerT	5′	GGT	GGA	GGC	GGC	TCT	GGT	GGC	GGT	GGC	AGT	GGC	GGC	GGT	GGA	TCC	3′

		S	G	G	G	G	S	G	G	G	G	S	G	G	G	G
LinkerB	5′	GGA	TCC	ACC	GCC	GCC	ACT	GCC	ACC	GCC	ACC	AGA	GCC	GCC	TCC	ACC	3′

					ApaLI		
pApaLI	5′	CCG	CCG	CCG	C*GT*	*GCA*	*C*TC	3′

					NotI		
pNotI	5′	GAG	TCA	TTC	T*GC*	*GGC*	*C*GC	3′

[a] Restriction site sequences on the primers are italicized. Linker sequences are underlined.

Maxisorp Loose microtiter strip (Nunc) were each filled with 100 μL of Pic–OVA diluted in PBS (100 μg/mL). Another three wells were each coated with 100 μL of OVA (100 μg/mL) to serve as negative controls. The strip was incubated at 4 °C for 16 h followed by blocking (200 μL/well) with milk (3%; Bio-Rad Laboratory Ltd., Hercules, CA) in PBS for 1 h at 25 °C. Phage suspension (10^{11} particles in 100 μL) was added to each well and incubated at 25 °C for 2 h. Wells were washed 20 times with sterile PBS containing 0.05% Tween-20 followed by 20 washes with PBS. Bound phage was eluted by incubation in sodium acetate buffer (0.1 M acetic acid and 0.15 M NaCl, pH 2.8) for 8 min followed by neutralization with 12 μL of 2 M Tris buffer (pH 9.5). Neutralized phage was used immediately to infect log phase *E. coli* TG1 cells (OD_{600} = 0.5) for 30 min at 37 °C followed by 30 min at 25 °C. The cells were then plated on 2xYT/tet agar plates and incubated at 30 °C for 16 h. Colonies were scraped from the plate and resuspended in 2xYT/tet broth to express phage as described in the previous section. Aliquots of concentrated phage were used for another round of panning and phage ELISA. In the second round of panning, the same concentration of Pic–OVA (10 μg/mL) was used to coat the wells of the microtiter strip.

To select for phage-displaying scFv with high affinity to picloram, the concentration of coating conjugate (Pic–OVA) used in panning was decreased to increase the stringency of selection. In the third and fourth rounds of panning, a lower concentration of Pic–OVA (10 μg/mL) was used to coat the wells of the microtiter strip. In the fifth round, 1 μg/mL Pic–OVA was used. Individual colonies from the fifth round of panning were grown separately in liquid culture to prepare the phage RF DNA for sequencing at the Laboratory Services Division, University of Guelph, Guelph, ON, Canada.

Soluble ScFv Expression. Sequence-specific primers were synthesized to introduce *Bbs*I and *Bgl*II restriction sites to the 5′ and 3′ ends of the scFv DNA, respectively, thereby facilitating directional cloning into the expression vector pSJF2 (kindly provided by National Research Council Canada, Ottawa, ON, Canada). The vector was derived from pUCE8 (21) and contains the *omp*A leader sequence for periplasmic expression, the c-myc tag for detection, and an His_5 tail for purification. Expression and periplasmic extraction of scFv were performed as described by Yau et al. (2). Crude extracts from each clone, which were directly used in ELISA, were stored at 4 °C in the presence of 0.1% protease inhibitor phenylmethanesulfonyl fluoride (PMSF; Sigma-Aldrich Chemical Co.).

ELISA. Phage-displayed and soluble scFv-based ELISAs were performed for screening and affinity determination, respectively. Pic–OVA (0.1 μg in 100 μL of PBS) was incubated in each well of a microtiter plate for 16 h at 4 °C. The plate was blocked with milk (3%, 200 μL) for 1 h at 25 °C. In the phage-display scFv-based ELISA, phage (100 μL, ~10^9 CFUs) in PBS was added to each well and incubated for 1 h at 25 °C. The plate was washed five times with PBS containing 0.05% Tween 20 (PBST). Anti-M13 Ab conjugated to horseradish peroxidase (Amersham Pharmacia Biotech Inc., Piscataway, NJ) was diluted 5000-fold in PBS according to the manufacturer's instructions. Diluted Ab (100 μL) was incubated in each well for 1 h at 25 °C. The wells were washed as described above. Substrate for the horseradish peroxidase was prepared

by dissolving 2,2′-azinobis(3-ethylbenzthiazoline)-6-sulfonic acid (10 mg; Sigma-Aldrich Chemical Co.) in 50 mM citrate buffer (25 mL, pH 4.0) containing 0.005% H_2O_2. The substrate (100 μL) was incubated in each well for 30 min, and absorbance (A) was measured at 405 nm using a microtiter plate reader (model 3550-UV, Bio-Rad). In the soluble scFv-based ELISA, anti-c-myc monoclonal Ab was used to detect the scFv, and goat anti-mouse Ab conjugated to horseradish peroxidase (Sigma-Aldrich Chemical Co.) was used to amplify the signal from anti-c-myc Ab.

CI-ELISA was performed to determine the affinity of the Ab to picloram (2). Prior to its incubation in wells coated with picloram conjugates, phage-displayed or soluble scFvs were preincubated with known concentrations of picloram, which competes with the coating conjugate for the antigen binding sites of the Ab. The signal (absorbance, A) detected was inversely proportional to the concentration of the picloram used. Standard inhibition curves were plotted with percent inhibition ($1 - A/A_0$, where A_0 is the average absorbance from wells not challenged with picloram) against the concentrations of picloram used in individual treatments. The affinity of the Ab in terms of IC_{50} (the concentration that inhibits 50% of the binding) was determined.

RESULTS AND DISCUSSION

Both polyclonal and monoclonal Abs have been widely used in immunoassays to detect a number of environmental pesticide contaminants (22, 23). However, the major obstacle to the use of Abs for residue analysis is the non-immunogenicity of small molecules and unsatisfactory affinity and specificity of antibodies resulting from poor immune response to hapten mimics (e.g., pesticide–protein conjugates) (22). Altering the characteristics of Ab at the protein level is labor intensive and technically more challenging than at the gene level. Recombinant Abs, therefore, provide an alternative to conventional polyclonal and monoclonal Abs. Cloning immunoglobulin genes from hybridoma cells secreting a pesticide-specific Ab has become routine and confirms the notion that recombinant Abs can replace polyclonal or monoclonal Ab in immunoassays (2, 5, 24, 25). Mutating cloned antibody genes can subsequently generate antibodies with improved affinity and specificity (20).

To date, the homogeneous nature of hybridoma cell cultures makes them the preferred choice as the source of genetic material for recombinant Ab production against agrochemicals, regardless of the requirements for a high level of technical skill and resources necessary for culture development and their maintenance. Bypassing the production of hybridomas is the immediate challenge for synthesis of recombinant Abs against small ligands (8). Desired Ab genes can be directly selected from splenocytes of immunized mice, provided

Picloram ScFv from Mouse Splenocytes

J. Agric. Food Chem., Vol. 49, No. 8, 2001   **3631**

that an effective and efficient selection technique, for example, phage-display technology, is available.

Many different phage-display systems allow the fusion of different types of Ab fragments to various phage proteins (*26*), such as gIIIp (*27, 28*), gVIp (*29*), and gVIIIp (*30*). With the development and improvement of affinity selection techniques, it is often difficult to decide which system is best for a particular purpose. Moreover, the quality of the selected Ab will be determined by the source of the genetic material, whether it is from unbiased (naïve), antigen-biased (hyperimmunized) (*31*), or synthetic libraries (*32, 33*). MacKenzie and To (*20*) reported that the constitutive gIIIp display with a phage vector fd-tet-GIIID was effective for enriching scFvs with the highest intrinsic affinities from a synthetic library.

Cloning Immunoglobulin Genes from Hyperimmunized Mouse. Seven days after the 12th boost with Pic−BSA, one of the four mice produced Abs with high affinities to picloram as determined by CI-ELISA. In the absence of free picloram, an A_{405nm} of 1.0 was obtained after the mouse anti-serum was diluted 5000-fold in PBS. In the presence of various concentrations of free picloram, the IC_{50} was determined to be 20 ppb. This mouse was sacrificed for splenocyte extraction 3 days after a final injection of Pic−BSA (see Materials and Methods).

A comprehensive repertoire containing a good representation of all the Abs present in the animal is critical to subsequent selection of Abs of choice using panning (*30*). Krebber et al. (*19*) developed universal primer sets for the amplification of V_H and V_L genes of mouse immunoglobulins and successfully amplified the genes using cDNA derived from various hybridoma cell lines of different specificities and family subtypes. In this study, the original restriction sites on the LB and HF primers were replaced with *Apa*LI and *Not*I, respectively (Table 1), for directional cloning into the phage vector fd-tet-GIIID (*20*). The V_L back primer mix (LB1-17 and LBλ) consisted of a total of 131 variants, all of which had the *Apa*LI site (Table 1). For the forward primers of V_L (LF1/2, 4, 5 and LFλ), the first repeat of the (Gly$_4$Ser)$_3$ linker DNA formed the 5′ end of all four members. Correspondingly, two sets of primers were used to amplify the V_H (Table 1). The first 15 nucleotides at the 5′ end of the V_H back primers (HB 1−19, representing a total of 94 variants) encoded the last repeat of the (Gly$_4$Ser)$_3$, whereas the four V_H forward primers (HF 1−4) contained the *Not*I site. From the amplification of V_H genes, a PCR product of 340 bp was obtained (data not shown). In contrast, amplification of V_L genes was problematic, and PCR products of ∼325 bp from four separate reactions were pooled together to obtain enough V_L for the subsequent linking reaction. On the basis of gene sequence analysis, which will be discussed later, the V_H of the clones selected after panning could be amplified using 16 of the 94 HB and 2 of the 4 HF primers. However, the V_L genes of all the clones selected could only be amplified by 4 of the 131 LB and 2 of the 5 LF primers. This result indicates that only a limited number of the V_L gene family members were rearranged to produce the picloram-specific antibody.

The next two rounds of PCR were performed to link the V_L and V_H genes using an overlap extension (*34*) of the nucleotide sequence of the flexible linker (Gly$_4$Ser)$_3$ (*35*). The linker primers LinkerT and LinkerB (Table 2), which encode the complete linker peptide in opposite directions, annealed to the partial linker sequences on the V_H and V_L genes, respectively. Therefore, the second PCR independently introduced the complete linker nucleotide sequence to both variable domains. In the third round of PCR, the two variable fragments were allowed to self-prime through the complementary regions of the linker nucleotide sequence, thereby joining the two fragments in the V_L−linker−V_H configuration. Subsequently, pApaLI and pNotI primers (Table 2) were used to amplify the complete scFv, producing a PCR product of 750 bp (data not shown). One advantage of this experimental design is that only the first PCR (V_H and V_L gene amplification from cDNA) requires degenerate primers. Both the second and third PCRs use single sets of primers, thereby preventing any biased amplification and the loss of diversity of the repertoire.

Construction of Phage-Display ScFv Library. The scFv DNA were inserted into the phage vector fd-tet-GIIID for the expression of phage-display scFv (PhscFv) (*20*). The use of this system allows the expression of scFv as fusion proteins to all five copies of gIIIp on the surface of the filamentous phage fd, compared to a phagemid/helper phage system (*8*), where only one to three copies of scFv are displayed. The phage vector has the advantage of a higher valency display, which improves the avidity for selection of low-affinity binders. Pentavalent display increases avidity, which is a prerequisite for the isolation of Abs with rapid dissociation kinetics from phage libraries. Without the avidity gain conferred by the pentavalent display of scFv by a phage vector, binders are lost in the washing steps designed to eliminate nonbinders (*20*).

The synthesized scFv DNA cloned into fd-tet-GIIID and transformed into *E. coli* TG1 resulted in a small library of 10^4 clones. We believe the library size was most probably limited by the efficiency of transformation after cloning as discussed by Sambrook et al. (*18*) and Amersdorfer and Marks (*31*). Nonetheless, MacKenzie and To (*20*) showed that stable and high-affinity lipopolysaccharide-specific scFv could be isolated from a library of similar size (i.e., 5×10^4). This system is, therefore, useful for constructing antigen-biased phage−Ab repertoires, from which most of the specific binders can be selected through panning because of their high avidity.
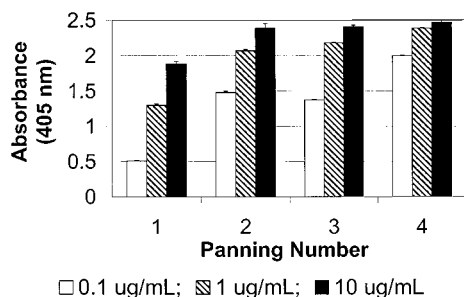
Phage-Displayed ScFv Specific to Picloram. Solid-phase panning as described by Griffiths et al. (*32*) was used to select picloram-specific PhscFv. Enrichment was subsequently determined by two indicators: increase in the numbers of phages recaptured after each round of panning and increase in the numbers of Pic−OVA binders detected by PhscFv-based ELISA.

Phages from the repertoire (∼10^{11} phage particles) were selected using Pic−OVA. Two rounds of panning were conducted in microtiter wells coated with 100 μg/mL of Pic−OVA. The number of phages recaptured after a second round of panning increased by >10-fold (Table 3). With the same amount of phage input and coating conjugate, the increased number of phages recaptured indicates the enrichment of Pic−OVA-specific PhscFv. However, this number was lower than reported values (*31, 32*). From an antigen-unbiased repertoire with 6.5×10^{10} members, Griffiths et al. (*32*) obtained an enrichment factor of up to 10^6-fold against human antigens (e.g., tumor necrosis factor) and foreign proteins (e.g., serum albumin), as well as a 20-fold enrich-

3632 *J. Agric. Food Chem.,* Vol. 49, No. 8, 2001

Tout et al.

Table 3. Enrichment of Phage-Displayed ScFv by Panning[a]

round of panning	coating conjugate concn (μg/mL)	no. of phages eluted[b] (×10³)
1	100	10
2	100	100
3	10	12
4	10	26
5	1	4

[a] Phage-display scFvs were selected against picloram conjugated to ovalbumin and reinfected into *E. coli* TG1. [b] Based on the number of colonies formed after reinfection of eluted phage particles to host bacteria.

Figure 1. ELISA to determine the specificity of phage antibodies to the herbicide picloram. Colonies recovered after each panning were pooled to produce phage-displayed scFv. Aliquots of the phage (~10⁹ CFU) were incubated in wells precoated with picloram conjugated to ovalbumin (Pic−OVA) and detected by anti-M13 antibody conjugated to horseradish peroxidase (see Materials and Methods). More phages were detected after each panning, demonstrating the enrichment phage-display scFv specific to Pic−OVA.

ment against haptens (e.g., fluoresein) after a single round of affinity selection. The lower enrichment factor obtained in our study may be related to the differences in nature and size of the original library used. In an antigen-biased library, because the majority of the members have certain specificities and affinities to the antigen of choice, a lower enrichment factor is anticipated.

The stringency of panning was increased in our third and fourth rounds of panning. The concentration of coating conjugate used was decreased 10-fold (10 μg/mL) to select Abs with higher affinities. As seen in Table 3, the number of phages recaptured first decreased 10-fold after the third round but increased thereafter, indicating further enrichment of picloram-specific PhscFv.

As stated earlier, the enrichment was also confirmed by PhscFv-based ELISA (Figure 1). All of the clones recovered after each round of panning were pooled together to produce PhscFv, which were examined for their activities toward Pic−OVA. The same number of phage particles was allowed to bind to different concentrations of Pic−OVA. The binding of the PhscFv collected from the same panning was directly proportional to the concentrations of Pic−OVA used. In addition, regardless of the concentration of the Pic−OVA used in the assay, more PhscFv bound to Pic−OVA after each panning. It was found that particular types of PhscFv, which bound specifically to Pic−OVA, were affinity-selected from the original repertoire and amplified through the selection−reinfection−propagation−reselection cycles.

In the fifth round, the stringency of panning was further increased (1 μg/mL of Pic−OVA) to retain the high-affinity binders. Following this panning, 20 ran-
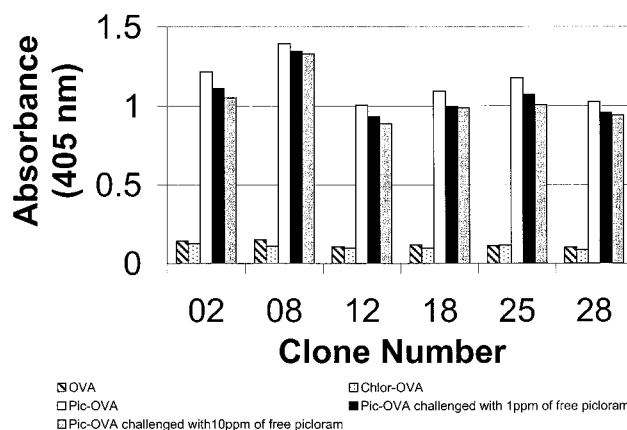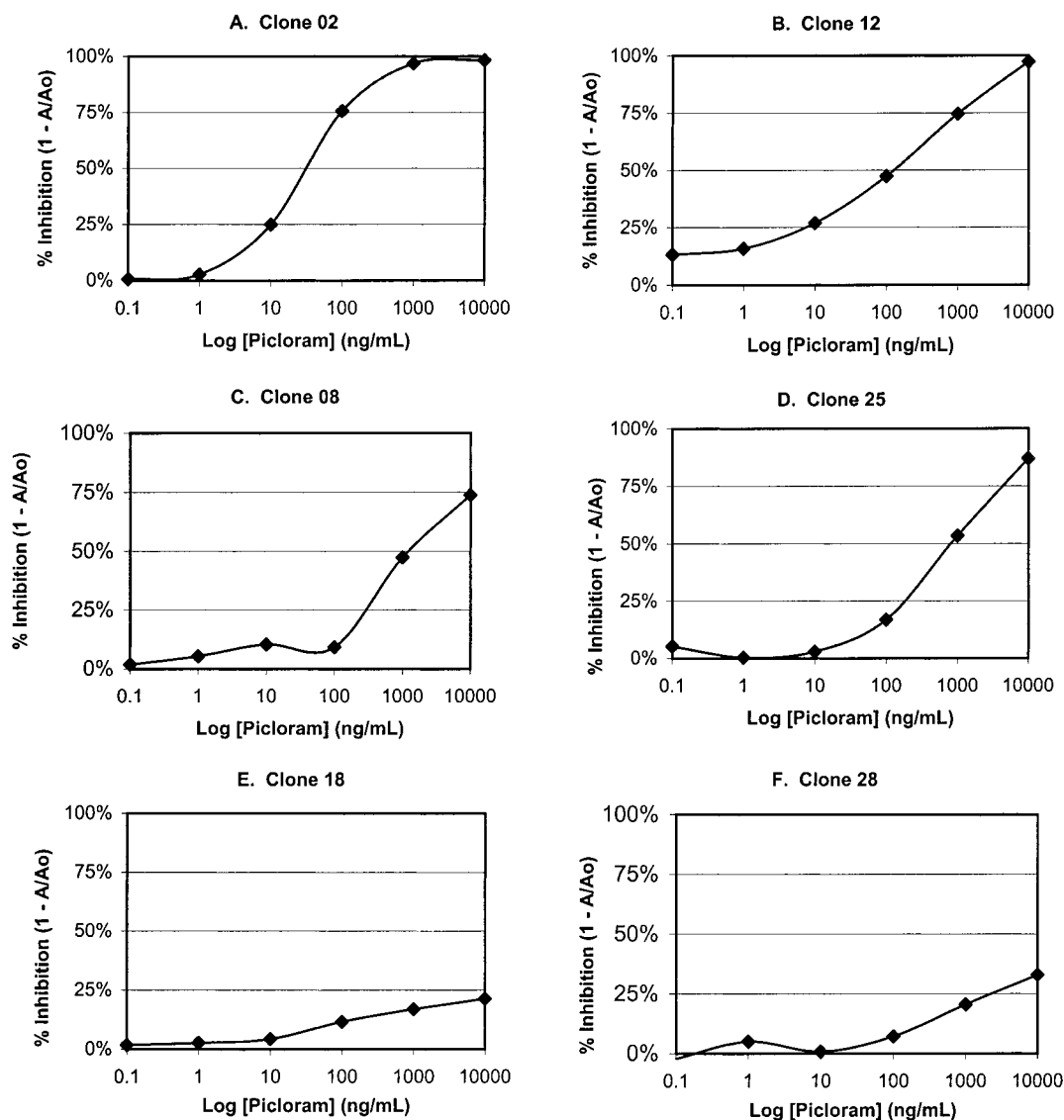

Figure 2. Competitive phage ELISA to determine the specificity of six individual clones recovered after the fifth round of panning. Six randomly selected clones were isolated, and phages from each clone were produced separately. Their binding to different coating conjugates (0.1 μg/mL), namely, Pic−OVA, OVA, and chlorimuron conjugated to OVA (chlor−OVA), was detected by anti-M13 antibody conjugated to horseradish peroxidase. All phages from different clones bound to Pic−OVA but not to OVA alone or to a herbicide from another chemical family conjugated to OVA (chlor−OVA). When the binding to Pic−OVA was challenged by the presence of free picloram at concentrations of 10 and 1 ppm, very little inhibition was detected as compared to the control (Pic−OVA).

domly selected clones were examined individually by PhscFv-based ELISA. Eighteen of the 20 clones tested showed different binding to Pic−OVA. Moreover, all 18 clones did not bind to OVA alone or to an unrelated herbicide (i.e., different chemical family) conjugated to OVA (chlorimuron−OVA) (data not shown). Despite the number of pannings (five rounds) with increasing stringency, nonspecific binders were present (~10%). For example, contamination of phage Ab libraries with recombinant vectors that have lost the scFv DNA occurs frequently (*36, 37*). Ab genes are usually amplified by PCR using degenerate primers. Mismatches and PCR errors will lead to point or out-of-frame mutations (*36, 38*). The mutated scFv−gIIIp fusion protein may cause vector instability, creating deletions in the Ab fusion genes. As a result, these clones carrying Ab-free vectors have a growth advantage over scFv-encoding clones and may contribute to the background levels of nonfunctional scFv molecules (*39*).

Six of the 18 positive clones (numbered 02, 08, 12, 18, 25, and 28) showing the highest affinities were further tested for binding specificity using CI-ELISA; however, when challenged with free picloram, very little inhibition was observed (Figure 2). Several authors (*1, 3*) have shown that antigen-binding characteristics of phage-displayed Abs are different than those of soluble fragments; that is, the PhscFv competes much more weakly than scFv for binding picloram in the CI-ELISA. The poor competition by PhscFv may reflect its increased avidity, in effect requiring 5 times more picloram molecules to saturate the antigen-binding sites. This effect may be overcome by decreasing the initial input of phages; however, the detection signal will be lower, resulting in reduced sensitivity of the assay.

Binding Characteristics of Soluble ScFv. In an attempt to overcome the poor inhibition of PhscFv binding by free picloram in CI-ELISA, six PhscFv clones previously selected were sequenced and subcloned into the pSJF2 vector and expressed as soluble scFv (hereafter referred to as clones 02, 08, 12, 18, 25, and 28).

Picloram ScFv from Mouse Splenocytes

J. Agric. Food Chem., Vol. 49, No. 8, 2001 **3633**

A. Clone 02

B. Clone 12

C. Clone 08

D. Clone 25

E. Clone 18

F. Clone 28

Figure 3. CI-ELISA to determine the affinity of soluble scFv from individual clones. Six clones identified from phage ELISA with potential specificity to picloram were subcloned into expression vector pSJF2. Recombinant scFv proteins were expressed into and extracted from periplasm of host bacteria. Crude extract was directly used in the assay (see Materials and Methods). Different affinities were detected in different clones with IC_{50} values ranging from 20 to 10000 ppb (ng/mL) (see text for specific IC_{50} values).

Crude periplasmic extracts prepared from these clones were tested by CI-ELISA for their binding properties to picloram. The estimated IC_{50} values were different for each clone. Soluble scFv clone 02 had the lowest IC_{50} value of 20 ppb (Figure 3A), which was comparable to that of recombinant Fab and monoclonal Ab previously prepared in our laboratory (*2*). Clones 08, 12, and 25 showed variable affinity to picloram with IC_{50} values ranging from 100 ppb to 1 ppm (Figure 3B−D). Clones 18 and 28 displayed minimal binding activity to picloram, with 10 μg/mL of picloram inhibiting these two latter scFv by only 25% (Figure 3E,F). Our results agree with those of others (*40, 41*) who report that PhscFv clones can be isolated from antigen-biased repertoires (i.e., hyperimmunized mice) and subsequently used to select soluble scFv binding to different epitopes with different affinities.

Our results show that the two forms of scFv, that is, PhscFv (Figure 2) and soluble scFv (Figure 3), although encoded by the same nucleotide sequence, do not have the same binding characteristics. This alteration in binding characteristics may be attributed to differences in the valency and folding of the antigen binding sites of the phage-bound versus the soluble scFv. Despite their differences, PhscFv and soluble scFv both have utility based on their applications. Divalent Ab molecules (e.g., monoclonal Abs) and monovalent Ab fragments (e.g., Fab and scFv) are very sensitive to inhibition by free analytes and make CI-ELISA a powerful tool for residue analysis (*1, 2, 5, 22*). The high avidity of the pentavalent PhscFv, in contrast, is valuable in securing hapten-specific binders with affinities that must be subsequently determined when expressed as soluble scFv.

Sequence Analysis. The scFv DNA of the six PhscFv clones were sequenced and their amino acid sequences deduced (Figure 4). All six sequences had different amino acid substitutions, and, as expected, most were in the complementarity-determining regions (CDR). The light chains of all six clones were slightly different from each other, but all belonged to the same $V\kappa 9A$ family joined to the same $J\kappa 2$ segment. As reported by others, Abs derived from the same library frequently have the same light chain segments (*41, 42*).

Clone	Heavy chain	Light chain
	FR1	
02	EVKLVESGGGLVKPGGSLKLSCAASGFTFS	DIQMMQSPSSLSASLGERVSLTC
08	EVMLVESGGGLVKPGGSLKLSCAASGFTFS	DIQMMQSPSSLSASLGERVSLTC
12	EVKLVESGGGLVKPGGSLKLSCAASRFTFS	DIQMMQSPSSLSASLGERVSLTC
25	EVKLVESGGGLVKPGGSLKLSCAASGFTFS	DIQMMQSPSSLSASLGERVSLTC
18	EVMLVESGGGLVKPGGSLKLSCAASGFTFS	DIQTMQSPSSLSASLGERLSLTC
28	EVMLVESGGGLVKPGGSLKLSCAASGFTFS	DIQTMQSPSSLSASLGERLSLTC
	CDR1	
02	SYAMS	RASQDIGSSLN
08	SFAMS	RASQDIGSTLN
12	SFAYS	RASQDIGSRLN
25	SDAMS	RASQDIGDSLN
18	SDTIS	RASQDIGDSLN
28	SLGTS	RASQDIGKGLN
	FR2	
02	WVRQTPEKRLEWVA	WLQQEPGGTIKRLIY
08	WVRQTPEKRLEWVA	WLQQEPDGTIKRLIY
12	WFRQTPEKRLEWVA	WLQQEPDGTIKRLIY
25	WVRQTPEKRLEWVA	WLQQEPGGTIKRLIY
18	WVRQTPEKRLEWVA	WLQQKPDGTIKRLIY
28	WVRQTPEKRLEWVA	WLQQKPGGTIKRLIY
	CDR2	
02	SISS-GG-STYYPDSVKGR	ATSSLDS
08	SISS-GG-STYYPDSVKGR	AASTLDS
12	YISG-GG-STYYPDSVKGR	ATSTLAS
25	SISG-GG-STYYPDSVKGR	AASTRDS
18	TISSGGG-STYYPDSVKGR	ASSTIYS
28	TISGGGGYSTYYPDSVKGR	ATSIVVS
	FR3	
02	FTISRDNARNILYLQMSSLRSEDTAMYYCAR	GVPKRFSGSRSGSDYSLTISSLESEDFVDYYC
08	FTISRDNARNILYLQMTSLRSEDTAMYYCAR	GVPKRFSGSRSGSDYSLTISSLESEDFVDYYC
12	FTISRDNARNIFYLQMSSLRSEDTALYYCAR	GVPKRFSGSRSGSDYSLTISSLESEDFVDYYC
25	FTISRDNPRNILYLQMTSLRSEDTAMYYCER	GVPKRFSGSRSGSDYSLTISSLESEDFADYYC
18	FTISRDNPQNILYLQMTSLKSEDTALYYCTR	GVPKRFSGSRSGSDYSLTISSLESEDFADYYC
28	FTISRDNARNIFYLQMTSLKSEDTATYYCAS	GVPKRFSGSRSGSDYSLTISSLESEDFVVYYC
	CDR3	
02	--GLLR-PDY	LQYASSPYT
08	--GYYR-PDY	LQYAGYPYT
12	-GTYYR-PDY	LQYALYPYT
25	LYGLFR-PDY	LQYALSPRT
18	-RDPYV-MDY	LQYASAAFT
28	--GYGRAMDY	LQYALVPRA
	FR4	
02	WGQGTTLTVSS	FGGGTKLEIKR
08	WGQGTTLTVSS	FGGGTKLEIKR
12	WGQGTTLTVSS	FGGGTKLEIKR
25	WGQGTTLTVSS	FGGGTKLEILR
18	WGQGTSVTVSS	FGGGTKLEIKR
28	WGQGTSVTVSS	FGGGTKLEIKR

Figure 4. Deduced amino acid sequences of V_H and V_L genes of six scFv isolated by phage-display from a spleen B cell repertoire of a mouse hyperimmunized with picloram conjugated to BSA. (Amino acids not conserved among the sequences are highlighted.)

Although the heavy chains of the six clones differed in their amino acid sequences, they all belonged to the same *V_H7183* family, which contains at least 19 characterized gene sequences in the genome of BALB/c mice (*43*). They were also joined to two different J segments. Clones 02 (with $IC_{50} = 20$ ppb), 08, 12, and 25 (the latter three with $IC_{50} > 100$ ppb) were joined to the J_H2, whereas clones 18 and 28 (minimal $IC_{50} > 10$ ppm) were joined to the J_H4 joining segments. As a result, the major variation of the six clones was found in the CDR3

of the heavy domains with differences in both the sequence and length. Both clones 02 and 08 have seven amino acid residues in the CDR3; clones 12, 18, and 28 have eight residues, and clone 25 has the longest CDR3 with nine residues. The theory of heavy chain dominance (*44*) specifies that producing high-affinity Abs in secondary response is dependent on the distinctive V_H-DJ_H rearrangements (CDR3, J_H) associated with somatic hypermutation at their CDRs. In other words, Abs selected from an unbiased library, especially for those

Picloram ScFv from Mouse Splenocytes

J. Agric. Food Chem., Vol. 49, No. 8, 2001 **3635**

hapten-specific ones, may have only low to moderate affinity (*32*).

There is significant variance present in the V_H7183 family. Therefore, it was difficult to conclude if the differences in the FR 1, 2, and 3 and CDR 1 and 2 of the variable domains of the clones isolated in this study were due to somatic hypermutations or whether they were just different members of the same family, or both (*45*). The V_H7183 along with the V_HQ52 families are located on chromosome 12 and grouped together at the 3′ end of the whole V_H gene cluster (*46*). B cells producing V_H genes from these two families are mainly found in bone marrow, neonates, or germ-free adult B splenocytes (*47*). Because of their proximity to the D and J segment genes, both families of genes are believed to be involved in early B cell development prior to any exposure of the immune system to foreign antigens. At the same time, Song et al. (*48*) found that the light chains are prominent in the primary encounter of immunogen of naive B cells. Serving as the crucial surface receptors on innate B cells, the light chains play an important role in inducing proliferation and differentiation into Ab-secreting plasma and memory B cells. These differentiated B cells were progressively affinity matured by somatic hypermutation throughout the prolonged immunization protocol. In our study, the immunogen used was a carrier protein (BSA) with complex structure bearing small molecules of the herbicide (picloram). At the beginning of the immunization there was very little specific binding of antisera to Pic−OVA even after the first eight injections (data not shown). The first encounter of the 10-week-old mice with the immunogen mainly triggered the immune response to the carrier protein, the immunogenic part of the conjugate. Subsequently, somatic hypermutation gradually improved the specificity of the Ab from the selected B cells to the hapten with increased affinity to the attached picloram molecules. Therefore, specific immune response to a small ligand is highly dependent on somatic hypermutation. This agrees with the notion that the affinity of scFv can be improved by in vitro maturation through mutagenesis and panning. Single rounds of mutagenesis and phage panning typically yield mutants with relatively small decreases in off-rates, and high-affinity mutants are generated in sequential mutagenesis/panning steps such as in the CDR walking strategy employed by Yang and colleagues (*49*).

Conclusion. We have successfully bypassed hybridoma technology to produce recombinant Abs with high affinity to a ligand of low molecular weight. Immunoglobulin genes were isolated from the spleen of a mouse hyperimmunized with the auxinic herbicide picloram (MW = 241.5). The V_H and V_L genes of the Abs were amplified, joined together by PCR, and cloned into the phage vector fd-tet-GIIID. Filamentous phage-displaying scFvs were selected against picloram and enriched through five rounds of panning. An scFv with an affinity comparable to that of the parent monoclonal Ab was isolated. Results indicate that herbicide-specific recombinant Ab fragments can be synthesized directly from hyperimmunized mice. DNA sequence analysis revealed that affinity maturation of antibodies specific to the herbicide picloram is dominated by somatic hypermutation.

The deduced amino acid sequences obtained from this study are useful in studying the structure and function of antibodies. The differences in the amino acid residues in these sequences may allow us to deduce the binding structure of the antibody to picloram, thereby allowing us to modify its affinity and specificity through site-directed mutagenesis. In the future, we will select from the library PhscFv specific to different auxinic herbicides from the pyridine family to which picloram belongs (e.g., clopyralid, fluroxypyr, and triclopyr). Compared to a panel of hybridoma cell lines, a panel of antibody fragments (i.e., scFv) with different affinities and specificities should be more easily derived and more versatile.

ABBREVIATIONS USED

CDR, complementarity determining region; FR, framework region; gIIIp, minor coat protein of filamentous phage; PhscFv, phage-display scFv; Pic−BSA, picloram conjugated to bovine serum albumin; Pic−OVA, picloram conjugated to ovalbumin; scFv, single-chain Fv; V_H, variable region on heavy chain; V_L, variable region on light chain.

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