Derivation and Properties of Recombinant Fab Antibodies to the Phenylurea Herbicide Diuron

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The Fab domain sequences from messenger RNA of a mouse hybridoma cell line secreting a monoclonal antibody (MAb) specific for the phenylurea herbicide diuron were amplified and inserted into the M13 phagemid vector pComb8. Phage displaying diuron-specific Fab fragments were selected by binding to magnetic beads coated with diuron hapten conjugates, and eluted with diuron. Soluble rFab in *E. coli* lysates bound diuron with half-maximal inhibition (I_{50}) of 1.6−12 ppb (ng/mL) in indirect and direct competition enzyme immunoassays (EIAs). The optimal competing hapten was different for indirect EIAs with proteolytic or recombinant Fabs and the intact MAb. Selectivity of rFabs for phenylureas was similar to that of the original MAb and its proteolytic Fab fragment and was not significantly affected by exchange of heavy and light chains among rFab clones, indicating that a single antibody species had been cloned.

Keywords: *Immunoassay; recombinant antibodies; monoclonal antibodies; diuron; herbicide; phenylurea; arylurea*

INTRODUCTION

Monoclonal antibodies (MAbs) have proven to be the reagents of choice for recovery and detection of small toxic molecules in many research and commercial applications (Karu, 1993). However, hybridoma technology requires substantial time, labor, expense, animal use, specialized cell culture facilities, and expertise to prepare and screen large numbers of cultures to select the best ones.

Interest in isolating and expressing antibody genes using recombinant DNA methods developed soon after the first descriptions of hybridoma production by Köhler and Milstein (Köhler and Milstein, 1975). Since 1989, recombinant antibody technology has evolved rapidly as a means of obtaining and manipulating immunoglobulin genes and expressing them in bacteria such as *E. coli* or in other hosts. A major advance was the development of bacteriophage M13 phagemid vectors. These may be propagated as phage that express on their coat the antibody encoded by their DNA. The desired antibodies can be selected by binding the phage to antigen using methods analogous to immunoassays (Barbas et al., 1991; Clackson et al., 1991; McCafferty et al., 1990).

Recombinant Fab (rFab) and single-chain Fv (ScFv) antibody fragments have several potential advantages over intact monoclonal antibodies (MAbs) for immunoaffinity recovery and analysis of small molecules. The rFabs are smaller than intact IgG molecules and may have superior physical or chemical properties. Diversity can be generated in vitro by making new combinations of heavy and light chains (Collet et al., 1992; Kang et al., 1991c; Marks et al., 1992) or by mutagenesis. Other functional groups such as a metal-binding or streptavidin-binding sequence may be added to the cloned sequence (Knappik and Plückthun, 1995; O'Shannessy et al., 1995). Antibody-enzyme gene fusions may be made (Ducancel et al., 1993) or variable-region genes from antibodies with different specificities may be cloned to express molecules called "diabodies" that are bivalent or recognize two different ligands (Holliger et al., 1993; Plückthun, 1992). Once the DNA sequence of a recombinant antibody is determined, the deduced amino acid sequence may be used to build a structure model based upon similarity to solve crystallographic structures. These models may be used to identify and change amino acids that affect the affinity and specificity of the antibody. It is also possible to express recombinant antibodies in other hosts such as insects, yeast, or higher plants. This may eventually lead to studies of how antibody expression may affect uptake or metabolism of small toxic molecules such as herbicides.

Considerable effort and resources are devoted to synthesis of haptens and hapten-carrier conjugates for use as immunizing antigens and competitors in immunoassays for small molecules. Mutagenesis and antibody engineering may become more predictable and cost effective than synthesis of new haptens and the uncertain outcome of immunization to create new specificities. Recombinant antibodies may be derived from cells of any species if appropriate oligonucleotide primers are available. Gene cloning from hybridoma cells offer the advantage that the parent antibody has been characterized and its messenger RNA (mRNA) should be relatively abundant.

The phenylurea herbicides provide a good model system for evaluating recombinant antibodies as useful reagents. Several phenylureas are used as herbicides worldwide. The compounds leach into groundwater, some of their metabolites are difficult to analyze, and instrumental analysis is expensive. Phenylureas inhibit

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photosystem II electron transport in bacteria, lower eukaryotes, and plants by binding to specific proteins (Oettmeier et al., 1982; Trebst et al., 1982). The submolecular interactions involved in this binding have been studied extensively, and phenylurea-specific antibodies may be an informative counterpart (Tietjen et al., 1991). We recently prepared MAbs and a sensitive enzyme immunoassay (EIA) for diuron, and we demonstrated its precision, accuracy, and reproducibility for monitoring residues in groundwater (Karu et al., 1994a,b). This report describes the derivation and properties of soluble rFab, consisting of the immunoglobulin V_H-C_{H1} (H) and V_L-C_L (L) sequences from one of the diuron hybridomas, cell line 481.

MATERIALS AND METHODS

Reagents. DNA restriction enzymes, T*aq* DNA polymerase, and T4 polynucleotide ligase were purchased from Promega Corp. (Madison WI). *E. coli* XL-1 Blue cells and M13K07 helper phage were from Stratagene Corp (La Jolla, CA). Buffer salts, agarose for DNA electrophoresis, nucleotides, antibiotics, ethidium bromide, isopropyl-β-thiogalactopyranoside (IPTG), and the redox dyes 5-bromo-1-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT) were from Sigma Corp. (St Louis, MO). All chemicals were analytical reagent or molecular biology grade, and all reagent solutions were prepared with glass-distilled water. Alkaline phosphatase-conjugated and unconjugated, affinity purified goat antimouse IgG were from Boehringer-Mannheim Corp. (Indianapolis, IN). Diuron I, II, and III haptens were generously provided by Drs. Marvin Goodrow and Bruce Hammock, University of California, Davis. The diuron I hapten has a carboxypropyl $(CH_2)_3$ spacer on the nitrogen closest to the phenyl ring. For one experiment in Table 1, a diuron I hapten with a carboxypentyl $(CH_2)_5$ spacer was used. The diuron II and III haptens have carboxypropyl and carboxypentyl spacers, respectively, on the nitrogen farthest from the phenyl ring (Karu et al., 1994a). Bovine serum albumin (BSA) and alkaline phosphatase conjugates of the haptens were prepared as described previously (Karu et al., 1994a). Bacterial culture media were purchased from Difco Laboratories, Detroit MI. The media and antibiotic resistance selection methods have been described by others (Barbas and Lerner, 1991; Kang et al., 1991a,b; Maniatis et al., 1989). DNA and RNA concentrations were determined by UV spectrophotometry.

Antigen-Coated Magnetic Beads for Phage Panning. Carboxylated paramagnetic polystyrene beads (cat. no. M7401 CN, 0.74 μm, styrene−6% divinylbenzene, 61.8% magnetite, in 10% aqueous suspension) were obtained from Bangs Laboratories (Carmel, IN). BSA conjugates of diuron I, II, and III haptens were covalently bound by a modification of the two-step procedure provided by the manufacturer. To make a bead suspension sufficient for about 50 pannings, 0.05 mL of the stock bead suspension (10% solids) was added to 0.43 mL of phosphate-buffered saline (PBS, 0.05 M $KH_2PO_4-K_2HPO_4$, pH 7.4, 0.15 M NaCl) containing 0.05% Tween 20 (PBST) in a 1.5 mL microfuge tube. The beads were sequestered on a magnetic concentrator (Stratagene Inc., La Jolla, CA), washed three times by resuspension in 0.5 mL of 0.1 M acetic acid−sodium acetate (pH 4.0), and derivatized by incubation (50 °C, 10 min) in 1 mg/mL of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (ECDI; Sigma E-6383) in the acetate buffer. They were then washed once in 0.05 M boric acid−sodium tetraborate (pH 9.0), resuspended in 0.43 mL of the borate buffer containing 0.2 mg/mL diuron I, II, or III−BSA conjugate (measured as BSA carrier), and incubated on a roller device at room temperature overnight. The conjugated beads were washed three times in 1 mL of sterile-filtered TPG buffer (50 mM NaH_2PO_4, 0.1% NaCl, 2 mg/mL gelatin, pH 6.6), resuspended in 0.5 mL of TPG buffer and stored at 4 °C, where they were stable for at least 1 month. For phage panning, aliquots (0.01 mL) of this suspension gave easily distinguishable pellets when sequestered in microfuge tubes.

The ability of the conjugated beads to bind diuron-specific antibody was verified by EIA. Beads coupled with unmodified BSA served as a negative control. Aliquots of 10−40 μL of bead suspension in 1.5 mL microfuge tubes were incubated with MAb 481 hybridoma culture fluid (1:200 in 0.09 mL TPG) on a roller apparatus for 1 h at room temperature. The beads were sequestered, washed three times with 0.5 mL of TPG, and resuspended in 0.1 mL of TPG containing alkaline phosphatase-labeled goat antimouse IgG (1:10 000; Sigma Chemical Co., St. Louis, MO). After 1 h on a roller at room temperature, the beads were collected and washed as before. Substrate solution (0.2 mL of 1 mg/mL *p*-nitrophenyl phosphate in 10% diethanolamine−HCl, pH 9.8, 0.4 mM $MgCl_2$, 3 mM NaN_3) was added. The tubes were incubated for 30 min at room temperature, after which the beads were sequestered and 0.1 mL aliquots of the supernates were taken into EIA wells for determination of absorbance at 405 nm.

Preparation of Monoclonal IgG and Proteolytic Fab Fragments. MAb 481, an $IgG_1\kappa$ immunoglobulin (Karu et al., 1994a), was purified from mouse ascites fluid to near-homogeneity by affinity chromatography on protein A-Sepharose columns using the high-ionic strength binding method described by Harlow and Lane (1988). Fab fragments were made by papain digestion of the pure IgG (1−2 mg/mL) using an Immunopure Fab Preparation Kit (Pierce Chemical Co., Rockford IL) according to the manufacturer's instructions. Recovery and concentration of the IgG and Fab were determined by absorbance at 280 nm.

Protein Electrophoresis and Western Blotting. Samples of MAb, proteolytic Fab, and bacterial lysates containing rFabs (10−20 μg of protein) were reduced with β-mercaptoethanol in 1% sodium dodecyl sulfate (SDS) and resolved by electrophoresis on 16% polyacrylamide gels (3 in. × 3 in. × 1.5 mm) in the presence of 0.2% SDS using standard methods (Harlow and Lane, 1988). The resolved bands were electrophoretically transferred to a poly(vinylidene difluoride) membrane (Immobilon PVDF, Millipore, Bedford MA) according to the gel apparatus manufacturer's instructions (70 mA constant current, 1 h, room temperature). Membranes were incubated in blocking buffer (PBST-1% Carnation nonfat dry milk) and probed with alkaline phosphatase-conjugated goat anti-Fab (Sigma, St Louis, MO; 1:5,000) or goat antimouse IgG (H+L) antibody (Boehringer Mannheim, Indianapolis, IN; 1:10 000) in blocking buffer. Bands that bound the alkaline phosphatase conjugate were visualized by color development with BCIP/NBT substrate (Harlow and Lane, 1988).

Isolation of mRNA and Synthesis of H and L Gene cDNAs. Standard molecular biology techniques (Maniatis et al., 1989) and previously described methods (Barbas and Lerner, 1991) were used to recover messenger RNA (mRNA) and selectively amplify and clone the H and L genes from diuron hybridoma line 481. Total RNA was isolated from 5×10^7 hybridoma cells, and the polyadenylated fraction (mRNA) was purified using a FastTrack kit (Invitrogen Corp., La Jolla, CA). Purity and amount of the RNA were determined by UV spectrophotometry. A complementary DNA (cDNA) copy of the mRNA was prepared with a U-Prime Kit (Pharmacia, Piscataway, NJ) using oligodeoxythimidylate (oligo-dT) primer.

PCR Amplification of H and L Genes. Sixteen individual polymerase chain reactions (PCR) were done concurrently on aliquots of the cDNA preparation using immunoglobulin-specific oligodeoxynucleotide primers described by Kang et al. (1991b) to amplify the H and L sequences. Reactions (0.1 mL) formulated as described by Kang et al. (1991b) contained *Taq* DNA polymerase and mixtures of a single 3′ H primer and one of nine 5′ H primers, or single 3′ L primer and one of six 5′ L primers. PCR was performed in thin-walled polypropylene tubes for 35 cycles (94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min) in a Microcycler E (Eppendorf, Inc., Fremont, CA). Products of the nine H reactions were combined and digested with restriction enzymes *Xho*I and *Spe*I. The desired fragments of approximately 650 base pairs (bp) were purified by agarose gel electrophoresis (Maniatis et al., 1989). Products from five of the six L reactions were pooled, digested with *Sac*I and *Xba*I enzymes, and fragments of approximately 650 bp were purified by agarose gel electrophoresis. The Lc2 5′ primer

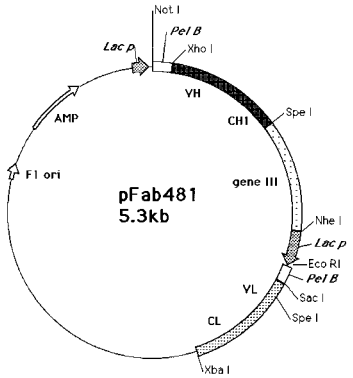

Figure 1. Map of the pComb3 vector containing the sequences for diuron rFab 481. Shown to approximate size are the V_H-C_{H1} sequence flanked by *Xho* and *Spe*I restriction sites, the sequence coding for M13 gene III protein with a *Nhe*I restriction site after the C-terminus, and the V_L-C_L sequence flanked by *Sac*I and *Xba*I restriction sites. Each of the antibody domains is preceded by a *PelB* leader sequence. Transcription is controlled by the *lacZ* promoters. The vector includes the gene conferring ampicillin resistance (amp) and the phage f1 replication origin (after Barbas and Lerner, 1991). The naturally occurring *Spe*I restriction site in the V_L sequence is also shown. Its precise location has been published (Bell et al., 1995b).

consistently yielded a PCR product about 100 bp smaller than the other products, so it was not used.

H and L Library Construction. A library of the H and L segments was cloned into the pComb8 phagemid. The steps may be visualized by referring to the phagemid map in Figure 1. Double-stranded circular DNA was isolated from *E. coli* XL1 Blue cells carrying pComb8. DNA purified by CsCl density gradient centrifugation and recovered by ethanol precipitation was cut with *Xho*I and *Spe*I, and the H DNA fragments were ligated into the vector (Barbas and Lerner, 1991; Kang et al., 1991b). This H library in pComb8 was purified on a CsCl density gradient, the DNA was recovered and digested with *Sac*I and *Xba*I, and the pooled L DNA fragments were inserted. The efficiency of L insertion into the vector was only about 50% based on agarose gel electrophoretic analysis (data not shown). This appeared to be due to inefficient action of the *Sac*I and *Xba*I enzymes on the PCR products. To exclude vectors that received only H, the entire preparation of vectors with inserts was treated with *Xho*I and *Xba*I, and fragments of approximately 1600 bp were purified by agarose gel electrophoresis. These were ligated into pComb8 DNA that was purified after digestion with *Xho*I and *Xba*I. This resulted in a library that either had both H and L or had no insert. This library DNA was used to transform *E. coli* XL1 Blue cells to produce phage for selection (Hanahan, 1985).

Selection of Diuron Hapten-Binding Display Phage. The H + L library was propagated as phage by infecting the transformed XL1 Blue culture with M13K07 "helper" phage. Phage from an overnight culture in 125 mL of super broth (10 g MOPS buffer, 20 g yeast extract, 30 g bactotryptone per liter, pH 7.0) was concentrated by precipitation with poly(ethylene glycol) 8000 and NaCl (Barbas and Lerner, 1991). The precipitate was recovered by centrifugation (12 000 × *g*, 20 min) and resuspended in 2 mL of PBS. Aliquots (0.05 mL) of phage suspension were added to 1.5 mL microfuge tubes containing 0.04 mL of TPG buffer and 0.01 mL of magnetic beads coated with diuron I−BSA or diuron III−BSA. The suspensions were incubated on a roller for 1 h at room temperature, and the beads were then sequestered with a magnet, resuspended in 1 mL of TPG with vortex mixing, and sequestered again. After two more washes in 1 mL TPG, the beads were suspended in 0.1 mL of TPG containing 1 μg/mL of diuron (1 ppm; 4.3 μM) and the tube was rolled 30 min at room temperature to competitively elute antibody-presenting phage. The beads were then sequestered and the supernate was transferred to a sterile tube and used to infect *E. coli* XL-1 Blue. Another 125 mL of culture was grown to amplify the selected phage, which were concentrated and selected again with the haptenated magnetic beads. Three cycles of selection and amplification were performed. The titers (concentrations) of phage applied to the magnetic beads and the phage recovered from each panning were determined by a standard plaque assay.

Identification of Diuron Hapten-Binding Clones. XL-1 Blue cells were infected with phage from each panning, and the phagemid-containing bacteria were grown on agar with 500 μg/mL of penicillin G. Colonies were transferred to sterile 0.45 μm nitrocellulose filters soaked in 10 mM IPTG. The filters were incubated overnight at 30 °C and then exposed to chloroform vapor for 20 min in a chromatography chamber to lyse the cells (Maniatis et al., 1989). The chloroform was allowed to evaporate, and the filters were gently shaken in a solution of 0.4 mg/mL lysozyme, 1 unit/mL pancreatic DNase I, and 30 mg/mL BSA in 0.05 M Tris-HCl (pH 8.0)−0.15 M NaCl−0.005 M $MgCl_2$ for 45 min at room temperature. This step was repeated using fresh solutions. The filters were then washed, blocked with 5% nonfat dry milk (NFDM) in PBST for 40 min (Barbas and Lerner, 1991), and incubated with diuron I−alkaline phosphatase (1:300 in PBST−5% NFDM) 2.5 h at room temperature. The filters were washed in PBST for 5 min five times, and colonies producing diuron-binding antibodies were visualized by adding the color development substrates BCIP and NBT (Dreher et al., 1991; Skerra et al., 1991). Positively staining colonies were recovered from the agar plates and propagated in broth. Aliquots were stored at −70 °C in broth containing 20% glycerol.

Transfer of rFab Genes to the pComb3 Vector. To transfer the H and L genes from the selected pComb8 clones into pComb3, the H DNA was excised from the pComb8 vector with *Xho*I and *Spe*I and the L sequence was released with *Sac*I and *Xba*I (Barbas and Lerner, 1991). These were gel purified and inserted into pComb3 DNA in two steps. A clone of pComb3 expressing a human rFab to tetanus toxoid (Barbas et al., 1991) was purified by CsCl density gradient centrifugation. The human H sequence was removed by cutting with *Xho*I and *Spe*I, and the vector DNA was purified by agarose gel electrophoresis. The mouse diuron H was ligated into pComb3 and amplified in XL-1 Blue, and the phagemid DNA was CsCl purified. This DNA was then cut with *Sac*I and *Xba*I and purified by gel electrophoresis, the mouse diuron L fragment was ligated in, and the product was again amplified in XL1 Blue and purified by CsCl gradient centrifugation. To verify the presence of the mouse H and L sequences, phagemid DNA was isolated from several colonies using a "magic miniprep" kit (Promega Corp.) and subjected to PCR with mouse 5′ H and 3′ L primers. Only clones that had murine H and L in the correct sized PCR product of about 1600 bp were amplified.

Preparation of Soluble rFab from pComb3. To produce soluble rFab that was not fused to the phage coat protein, it was necessary to excise the M13 gene III sequence from pComb3 and religate the vector (Barbas and Lerner, 1991). In theory the gene III sequence could have been excised in one step by concurrent digestion with *Nhe*I and *Spe*I, which create compatible ends that can be ligated. However, trial digests revealed an unexpected *Spe*I site within the 664 bp mouse diuron L sequence. Consequently, gene III had to be excised in two steps. First, the clone was cut with *Xho*I and *Spe*I, creating three fragments: the intact H (approximately 0.7 kb), a fragment of about 1.6 kb (presumably extending from the end of the H to the second *Spe*I site in the L), and the remainder of the pComb3 vector. In a second reaction, the phagemid DNA was digested with *Xho*I and *Nhe*I, resulting in a fragment from the start of the H through the end of the gene III sequence, and the remainder of the vector (including the intact L sequence). The H fragment from the first digestion was purified from agarose gels and ligated into the gel-purified large fragment from the second digestion to give an intact H+L−pComb3 clone lacking gene III, which would express soluble diuron rFab.

XL-1 Blue cells transformed with this construct were grown overnight at 30 °C in 100 mL of super broth with 1 mM IPTG

to induce soluble rFab expression controlled by the *lac* promoters. The cells were collected by centrifugation and resuspended in 1−2 mL of 20% (w/v) sucrose−0.05 M Tris-HCl (pH 7.5). The culture medium was freeze-dried and resuspended in 0.01 volume of distilled water. Whole cell extracts were prepared by resuspending the cells in 0.02 M Tris-HCl−10^{-4} M phenylmethylsulfonyl fluoride (PMSF) and sonicating with a microprobe (Branson Corp., Danbury CT) for five 15 s pulses at 50−60% maximum power or by five consecutive cycles of freezing and thawing. Solids were removed by centrifugation (12 000 × *g*, 10 min), and the supernates were used in assays. Periplasmic lysates were prepared with lysozyme and Brij 58 surfactant (Karu and Belk, 1982), and solids were removed by centrifugation. The lysates, which contained 1 mM dithiothreitol and 0.2 mM PMSF, were stable at −20 °C for at least 3 months.

EIA Procedures. Indirect and direct EIAs were performed as previously described (Karu et al., 1994a) except that the blocking solution and diluent for bacterial extracts, standards, alkaline-phosphatase−goat antimouse Fab conjugate, and diuron−alkaline phosphatase conjugate were PBST−OA (PBS, pH 7.2 containing 0.05% Tween 20 and 1% ovalbumin). For direct EIAs the wells were coated with 20 ng of affinity purified goat antimouse Fab (Boehringer−Mannheim). The diethanolamine−HCl alkaline phosphatase substrate buffer was supplemented with 2 mM K_2HPO_4, which abolished *E. coli* alkaline phosphatase activity from the bacterial extracts. Competitive indirect and direct EIAs were done using phenylurea reference standards >98% pure (AccuStandard Inc., New Haven, CT). The rate of color development (absorbance at 405 nm) was recorded and dose−response curves were fitted with the four-parameter logistic equation using Passage II software on a Macintosh computer.

RESULTS

Derivation of the Recombinant Fabs. Diuron-specific hybridoma line 481 was used as a source of IgG_1k mRNA for which there were published PCR primers (Kang et al., 1991b). This hybridoma was originally derived by fusing a Balb/C mouse myeloma with splenocytes from Biozzi mice (Biozzi et al., 1979; Karu et al., 1994a). We assumed that the mRNA for MAb 481 would be an abundant immunoglobulin gene transcript in these cells. In two earlier experiments, we extracted mRNA from splenocytes of Biozzi mice immunized with diuron II−BSA conjugate. In both cases we were unable to recover diuron-specific rFab sequences with the primers described by Kang. This may have been because other IgG mRNAs (not diuron-specific) were much more abundant in the splenocyte extracts and reduced the probability of cloning and selecting a diuron-specific antibody.

Pools of electrophoretically purified, restriction enzyme-digested PCR products were used to construct H and L libraries in the pComb8 vector, which expresses the rFab as a fusion with gene VIII protein, the major phage coat protein. This results in display of 5−40 copies of the rFab along the entire phage particle (Kang et al., 1991a). In our first attempts to select phage displaying diuron hapten-binding rFabs from the pComb8 clones, we used microplate wells coated with diuron I− or III−BSA and a phage-panning protocol first described by Parmley and Smith (Parmley and Smith, 1988) with modifications (Barbas and Lerner, 1991). Phage were recovered by elution with 0.1M glycine−HCl (pH 2.2). However, we were unable to increase the proportion of hapten-binding phage over several rounds of panning by this procedure. The alternate procedure described in Materials and Methods, using hapten conjugate-coated magnetic beads and elution with diuron, yielded a 10−100-fold enrichment of hapten-binding phage in three cycles of panning and amplification. After each round of panning, bacteria were infected with the recovered phage and grown as colonies on agar. Colonies expressing specific rFab were identified with the blot technique described in Materials and Methods. All the colonies, including uninfected controls, showed some nonspecific staining, possibly due to entrapment of the conjugate by the bacterial proteins or the presence of *E. coli* alkaline phosphatase. To reduce the background, two consecutive blots were made from each Petri plate. In each case the second blot received less bacterial protein and had a lower background than the first one. However, despite the increase in titer of phage specifically eluted by diuron, only 4 of 50 colonies tested developed a much darker, more uniform stain than the others (data not shown). These were selected as likely to be producing diuron-binding rFab−gene VIII fusion protein. One isolate, designated no. 1, was obtained from the first round of panning on beads coated with diuron I−BSA. The other three, designated 2, 3, and 4, were selected from the first or second panning on beads coated with diuron III−BSA.

Transfer of H and L Sequences from pComb8 to pComb3. During the course of this work the pComb3 vector became available. pComb8 produces the H and L as a dicistronic mRNA transcribed from a single promoter (Kang et al., 1991a). Phage produced with pComb8 display numerous copies of rFab as fusions with the gene VIII coat protein all along the surface of the phage. The transcription efficiency may be better in pComb3, where the H and L sequences are each transcribed from a separate promoter as monocistronic mRNAs (Barbas et al., 1991; Kang et al., 1991a). With pComb3 the rFab forms as a fusion with the M13 gene III protein, with only one to three copies on the tip of the phage filament (Barbas and Lerner, 1991). However, we used pComb3 primarily as a means to overexpress soluble rFab. To do this, we excised the H and L sequences from the four diuron hapten-binding clones in pComb 8 and inserted them into pComb3. Figure 1 is a map of the complete vector containing the mouse diuron antibody sequences.

Identification and Avoidance of a *Spe*I Restriction Site in the L Chain. Our initial attempts to convert the recombinant pComb3 phage to phagemids that would produce soluble rFab were unsuccessful because of an unexpected *Spe*I cleavage site near the middle of the mouse diuron L sequence. To circumvent this, we electrophoretically purified the H and L sequences in two steps and rejoined them as described in Materials and Methods so that the L chain DNA was not exposed to the *Spe*I enzyme while the gene III sequence was removed. The vectors reconstructed by this procedure produced soluble rFab.

Recovery of Soluble rFab. Soluble rFab may be released into the culture medium, retained in the periplasmic space, or reside in the bacterial cytoplasm. To determine how rFab was distributed, extracts were prepared as described in Materials and Methods, and antibody titers were measured by indirect EIA on wells coated with the diuron−BSA conjugates. Very little rFab was detected in the concentrated culture medium. Whole cell lysates made by freezing and thawing or sonication contained significant amounts of rFab, but nucleic acids and other material in the extracts made them viscous and caused interference in EIAs. The lysozyme−Brij periplasmic lysates contained no interfering material. Dilutions from 1:10 to 1:500 gave

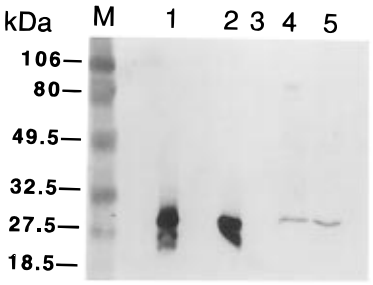

Figure 2. Resolution of MAb 481, proteolytic Fab, and rFab. Samples were separated by SDS−polyacrylamide gel electrophoresis, electrophoretically transferred to PVDF membrane (Immobilon, Millipore Corp., Bedford, MA), and visualized with alkaline phosphatase-conjugated goat antimurine Fab as described in Materials and Methods. Intact H chains did not react with the goat antimurine Fab conjugate used for this experiment. The samples were (M) molecular size markers as indicated, (1) proteolytic Fab 481, (2) purified IgG 481, (3) periplasmic lysate from IPTG-induced *E. coli* XL-1 Blue that was not transformed with the pComb3 phagemid, (4) periplasmic lysates from IPTG-induced XL-1 Blue transformed with rFab isolate 2.1, and (5) periplasmic lysate from IPTG-induced XL-1 Blue transformed with rFab isolate 1.5.

reproducible, strong dose responses on wells coated with diuron II− or diuron−III BSA but no binding above background on wells coated with diuron I−BSA or unconjugated BSA.

Comparison of MAb, Fab, and rFab. Purified IgG and Fab fragments were made from MAb 481 and analyzed by SDS−polyacrylamide gel electrophoresis as described in Materials and Methods. The IgG and proteolytic Fab fragments were essentially homogeneous on Coomassie blue-stained gels. The apparent molecular weight of the intact H chain was approximately 53 kDa. The truncated H chain of the rFab (V_H-C_{H1}) and the L chain were about 23 kDa and could not be distinguished from each other. Subsequent DNA sequencing showed that the V_L-C_L and V_H-C_{H1} polypeptides were 218 and 223 amino acids long, respectively (Bell et al., 1995b). In a western blot probed with alkaline phosphatase-conjugated goat antimouse whole-molecule IgG, the lysates from three separate isolates of rFab had a broad, heavily staining band at 22−25 kDa and numerous fainter bands from 40 to 200 kDa. A western blot probed with alkaline phosphatase-conjugated goat antimouse Fab showed bands of the same size in the rFab isolates, IgG 481 L chain, and Fab fragments derived from the MAb by papain digestion (Figure 2).

Hapten Preference. In the initial screening the soluble rFab showed a strong preference for binding to diuron II− and diuron III−BSA conjugates rather than diuron I conjugates in the indirect EIA. Proteolytic Fabs prepared by papain digestion of MAb 481 IgG showed the same binding preference as the rFabs (Table 1). By contrast, intact MAb 481 ascites fluid and purified IgG showed a preference for binding diuron I−BSA (Karu et al., 1994a). The rFabs also bound weakly to a diuron I conjugate with a five-carbon spacer (Table 1). In a direct EIA, MAb 481 and two of three rFab isolates all bound diuron I− and diuron III−alkaline phosphatase (Table 2) with roughly equal efficiency. Thirteen separate isolates of the rFabs competitively bound diuron in indirect EIAs with half-maximal inhibition between 2.5 and 11.6 ppb, while MAb 481 had an I_{50} value of about 500 ppb on wells coated with diuron III−BSA (Table 3). Thus, the indirect EIA with rFab and diuron III−BSA coating was approximately as sensitive as with the parent MAb and diuron I−BSA coating. The differences in I_{50} and slope values may have been due partly to differences in rFab expression and concentration between the lysates and were not significant. Similar I_{50} values were obtained from the direct competition EIA with diuron I−alkaline phosphatase.

Specificity for Phenylureas. Cross-reactivity with other phenylureas was tested by indirect competition EIA. MAb 481 was approximately 3% cross-reactive with both monuron and linuron when the competing

Table 1. Binding of MAb 481, Proteolytic Fab, and rFabs to Diuron Conjugates in the Indirect EIA

		diuron hapten−BSA coating conjugate EIA rate ($\Delta A_{(405\ nm)}$/min × 10^{-3})		
antibody	dilution[e]	I $(CH_2)_3$	I $(CH_2)_5$[f]	III $(CH_2)_5$
MAb 481 ascites[a]	10^8	36	nt	15
MAb 481 IgG[b]	10^6	18	nt	6
MAb 481 Fab fragment[c]	400	13	nt	92
rFab 1.6[d]	100	4	3	42
rFab 2.8[d]	100	2	0	34
rFab 3.5[d]	30	2	0.1	31

[a] Raw mouse ascites fluid clarified by centrifugation. [b] Purified from ascites fluid by protein A−sepharose chromatography. [c] Prepared by papain digestion of purified IgG as described in text. [d] Periplasmic lysates of *E. coli* as described in text. [e] Largest amount that bound to wells coated with 1 μg of diuron III−BSA without saturating the coating. [f] Diuron hapten with a five-carbon spacer on the nitrogen closest to the phenyl ring. nt = not tested.

Table 2. Reaction of MAb 481 and rFabs in the Direct EIA

		diuron−alkaline phosphatase conjugate EIA rate ($\Delta A_{(405\ nm)}$/min × 10^{-3})	
antibody	dilution[c]	I $(CH_2)_3$	III $(CH_2)_5$
MAb 481 culture fluid	300	106	109
MAb 481 ascites[a]	5000	110	98
rFab 1.6[b]	100	22	2
rFab 2.8[b]	100	38	55
rFab 3.5[b]	20	27	31

[a] Raw ascites clarified by centrifugation. [b] *E. coli* periplasmic lysates as described in text. The nomenclature 1.6 refers to rFab isolate no. 1, colony no. 6, etc. [c] Dilutions that saturated wells coated with 20 ng of a trapping antibody (goat antimurine Fab).

Table 3. Dose Response Parameters for MAb 481, Proteolytic Fab, and Independent Isolates of rFabs Determined by Indirect Competition EIA on Wells Coated with Diuron III−BSA

antibody	I_{50} (ppb diuron)[b]	slope[b]
MAb 481	505	2.4
Fab 481	1.8	0.9
rFab 1.5[a]	4.1	1.2
1.6	6.0	1.2
1.7	4.0	1.3
1.8	2.9	1.4
2.3	4.9	1.5
2.8	3.6	1.1
2.9	4.0	1.2
2.10	2.5	1.2
3.2	6.6	1.3
3.4	9.0	1.1
3.5	11.6	1.4
3.6	9.5	0.9
3.8	9.2	1.3

[a] The rFabs were expressed from pComb3 after gene III was deleted. The isolate number is to the left of the decimal. The number to the right of the decimal designates the bacterial colony that was used. Isolate no. 4 was not tested. [b] Values are the mean for four-parameter logistic fits of three replicates tested with eight concentrations of diuron.

Table 4. Specificity of rFabs for Various Phenylureas in Indirect Competition EIA

		I_{50} (ppb)		
rFab[a]	coating conjugate	diuron	monuron	linuron
1.6	diuron III−BSA	8.2	89.6	214
1.8	diuron III−BSA	3.4	57.6	110[b]
2.3	diuron III−BSA	3.0	45.6[b]	157[b]
2.10	diuron III−BSA	2.8	24.1[b]	110[b]
3.2	diuron III−BSA	6.7	65.3	148
3.5	diuron III−BSA	7.6	82.5[b]	265[b]

[a] The rFabs showed no appreciable binding to diuron I−BSA. I_{50} and slope values on wells coated with diuron II−BSA were similar to the results shown for diuron III−BSA. [b] Estimated by graphic interpolation. All other values were obtained by iterative four-parameter fitting.

hapten was diuron I. This was consistent with our previous results (Karu et al., 1994a). Six independent isolates of the rFabs were 3−10% cross-reactive with monuron and 2−5% cross-reactive with linuron when the competing hapten was diuron III (Table 4). Neither MAb 481 nor the rFabs bound fenuron, which has no chlorines.

Chain Shuffling. Although the diuron rFabs were derived from a single hybridoma cell line and had similar properties in the EIAs, it was possible that they might contain different H or L domains due to somatic mutation or errors introduced by PCR or cloning. To test for this, the H and L inserts were excised from the four original constructs, introduced back into the pComb 3 vector in 12 new combinations, and expressed as soluble rFabs. This is known as chain shuffling (Kang et al., 1991c). Because of the *Spe*I site in the L sequence, the H and L were isolated and ligated into the vector as described for preparation of soluble rFab in Materials and Methods. The new combinations were designated with numbers indicating the source of the H and L, respectively. For example, rFab 23 was composed of the H from rFab 2 and the L from rFab 3. The chain-shuffled rFabs and the original isolates showed comparable patterns of binding to diuron I−, II−, and III−BSA (Table 5). Several that were tested for competitive binding of diuron, monuron, and linuron gave I_{50} values similar to the original isolates in the indirect EIA (data not shown). Thus, there were no significant functional differences among the chain-shuffled rFabs.

Solvent Tolerance. We tested the effect of methanol on binding of pure IgG, proteolytic Fab, and rFab to diuron III−BSA coating antigen because methanol is an effective solvent for recovery of diuron residues from C_{18} solid-phase extraction columns (Karu et al., 1994b). Figure 3 illustrates the progressive loss of hapten binding by proteolytic Fab and rFab in increasing amounts of methanol. Binding of intact IgG was unaffected or slightly improved over this range of methanol concentrations. Coated wells incubated with up to 30% methanol for 1 h prior to addition of antibody showed no change in ability to bind Fab or rFab (data not shown). This indicated that the methanol affected the antibody and not the coating conjugate.

DISCUSSION

We used immunoglobulin-specific PCR primers and phage display methods to clone the H and L sequences for a diuron-binding antibody and express it as a Fab fragment in *E. coli*. Using a hybridoma as the source of antibody genes has the practical advantages that the

Table 5. Binding of Original and Chain-Shuffled rFab Isolates to Diuron Hapten Conjugates in the Indirect EIA

	coating conjugate $\Delta A_{(405\ nm)}/min \times 10^{-3}$		
rFab[a]	diuron I−BSA	diuron II−BSA	diuron III−BSA
1.5	0.1	21.5	36.4
2.3	0	20.7	32.8
3.2	0	7.1	4.8
12.1	0	23.6	39.8
13.1	0	8.7	17.6
14.1	0.8	12.8	28.9
21.1	0	24.2	42.9
23.1	0	7.8	14.4
24.3	0	13.4	28.0
31.1	0	14.0	24.6
32.2	0	16.6	44.3
34.1	0.4	14.5	26.6
41.1	1.5	17.9	38.3
42.1	0	1.6	7.9
43.1	0	11.0	21.5
44.2	0	10.1	32.2

[a] Numbers to the left of the decimal indicate the rFab isolate in pComb3. Double digits indicate rFabs with shuffled H and L from two isolates. Numbers to the right of the decimal denote individual colonies of the clone. For example, 24.3 is rFab from colony 3 of a clone with shuffled H and L from original isolates 2 and 4.

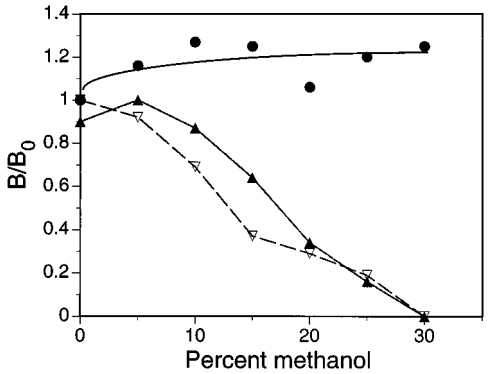

Figure 3. Effect of methanol on antibody binding to diuron III−BSA. Microplate wells were coated overnight with 500 ng of diuron III−BSA as described in Materials and Methods. Samples of purified MAb 481 IgG (●), Fab made from the IgG by digestion with papain (▲), and rFab 3.5 (▽) in 0.1 mL of PBST containing the indicated amounts of methanol were added to the wells. After 1 h at room temperature the wells were washed, and alkaline phosphatase−goat antimouse Fab was added in PBST for 2 h at room temperature. The remainder of the EIA was conducted as in Materials and Methods. Results are expressed as the ratio of color development rates obtained with the sample (B) to that obtained with the same antibody in PBST with no methanol (B_0).

MAb's performance characteristics have been well studied. We also assumed that the predominant mRNA species should be the H and L transcripts for that antibody. However, this assumption has been shown to be incorrect in studies with some hybridomas and all of the myeloma lines commonly used as fusion partners (Alt et al., 1980; Kwan et al., 1981; Perry et al., 1980). Myelomas such as P3X63AG8.653 (which was the fusion partner for producing diuron hybridoma 481) do not produce a L protein. However, they express an aberrant κ L transcript in amounts that may exceed that of the desired L mRNA (Cabilly and Riggs, 1985; Carroll et al., 1988). The aberrant κ L transcript codes for tyrosine instead of the highly conserved cysteine at position 23 that is important for intrachain disulfide bonding (Carroll et al., 1988). Undesired transcripts may also come from the splenocyte partner in a fusion (Cabilly and

Riggs, 1985). These would be present in the cDNA made from the total mRNA population and likely to be amplified by immunoglobulin-specific PCR primers. The rFab clones that we selected all contain functional L sequences. They have a *Spe*I restriction site not found in other L sequences, and the cysteine at position 23 is conserved (Bell et al., 1995a,b).

The rFabs were the same size and functionally identical with Fab fragments made by papain digestion of MAb 481, although both the recombinant and proteolytic Fabs behaved differently from the full-sized MAb 481 in assays. However, the rFab fragments may not be perfect copies. The first four amino acids at the N-termini of the V_H and V_L sequences were derived from the pComb3 vector. These codons were conserved in the 5′ PCR primers to retain the cloning site. They may not be the same or have the same conformation as the four N-terminal amino acids that occur naturally in MAb 481. To determine this, it would be necessary to analyze the N-terminal amino acid sequences of MAb 481 H or to clone and sequence a cDNA containing the V_H domain without using PCR. We did not do this because the rFabs and proteolytic Fabs performed identically in our asssays.

The rFabs bound diuron II and III hapten conjugates and competitively bound free diuron similarly to proteolytic Fab fragments of the parent MAb. Several independent isolates of the rFabs, as well as rFabs with shuffled H and L sequences, reacted similarly with diuron and monuron in competition EIAs. The relatively small difference in cross-reaction with linuron may have been due to differences in competition with the diuron III hapten. The results indicated that we selected a single functional combination of V_H and V_L domains from the amplified sequences. We subsequently found that the DNA sequences of two independent rFab isolates differed by only a single nucleotide that codes for the same amino acid near the C-terminus of the C_L domain (Bell et al., 1995a,b). This "silent" difference may have arisen during the gene cloning or it may reflect somatic mutation in the mRNA recovered from the hybridoma. Selection by an antigen-binding method such as phage panning or colony blots is necessary to recover functional rFabs because artifacts may arise in gene cloning. These include the presence of cryptic immunoglobulin gene transcripts and errors introduced in amplification by PCR, as well as somatic mutation.

The greater sensitivity of Fab fragments to organic solvent could limit their usefulness for practical assay applications. We do not know whether the increased solvent sensitivity is localized to the combining site or reflects a more general change in the rFab structure. Recently, some investigators have modified recombinant antibody structures by directed mutagenesis to make them more resistant to denaturation by heat and organic solvent (McCartney et al., 1994; Reiter et al., 1994).

Three major problems were encountered in the derivation of these rFabs. First, the phage were not selectively recovered by published panning methods using hapten conjugates immobilized in microplate wells and low-pH elution. A modified protocol using paramagnetic beads as the solid phase and diuron as the eluant was much more efficient. Second, a *Spe*I restriction site one-third of the way into the L sequence necessitated a change in the strategy for removing the M13 gene III sequence so that soluble rFab could be expressed. No *Spe*I sites had previously been reported in other antibody DNA sequences cloned into pComb8 or pComb3 or in a search of 50 L sequences from a large immunoglobulin sequence database (Chaudhary et al., 1990). Nevertheless, *Spe*I recognizes a 6-bp sequence (5′-ACTAGT-3′) that would be statistically expected to occur once in every 4096 base pairs. Inconvenient restriction sites can be detected by electrophoretic analysis of trial restriction digests and circumvented by modifying the cloning protocol.

The third unexpected finding was that different hapten conjugates were optimal for binding intact IgG and Fabs in the indirect EIA. The effect was less evident in the direct EIA format. Hapten recognition by all soluble rFab clones was similar to that of Fab fragments made by proteolysis of purified 481 IgG but not to that of full-sized 481 IgG. One possible explanation is that the rFab−gene III protein fusion on the phage had steric properties that resembled those of full-sized IgG 481. We might have discovered this before we began gene cloning if we had made proteolytic Fab fragments and analyzed their binding to different conjugates. As with production of MAbs, it is valuable to have more than one screening antigen and assay format when deriving rFab and single-chain Fv antibodies.

The membrane blot method of screening antibody expression in transformed colonies had several drawbacks. It was difficult to screen large numbers of colonies; substantial amounts of hapten−enzyme conjugate were required, and the signal-to-noise ratio was poor. The transformed cells expressed rFab−gene VIII fusion protein. We screened the blots only with diuron I−alkaline phosphatase, assuming that the fusion protein would bind the hapten conjugate similarly to MAb. The subsequent experiments showing that soluble rFab preferentially bound the diuron II and III haptens suggested that this assumption was incorrect. This may explain why the colony blot screening did not show the enrichment of positives expected from the increased phage titer with successive rounds of phage panning. Use of peroxidase conjugates may be an alternative to improve the signal-to-noise ratio (Dreher et al., 1991), but hapten recognition by soluble recombinant antibodies and fusion proteins may be difficult to predict. Screening methods such as an EIA to detect phage that display specific antibodies may be more definitive (Kingsbury and Junghans, 1995).

In addition to their uses in immunoassay and sensor formats for analytical purposes and immunoaffinity applications for residue recovery, recombinant antibodies potentially may be valuable for detoxification and facilitated breakdown of toxic chemicals or for conferring herbicide resistance. For example, rFabs expressed in plants may be able to block or compete with binding of diuron to its target sites in the electron transport systems. Conversely, the antibody might facilitate sequestration of diuron and thus potentiate its effects in the plant. Biologically active antibody expression has been demonstrated in plants (Hein, 1990; Hiatt, 1989; Hiatt et al., 1989; Owen et al., 1992; Taviadoraki et al., 1993), but to date, antibody-mediated herbicide resistance or sensitivity has not been demonstrated. Tobacco lines resistant to diuron have been developed (Rey et al., 1990), and the molecular genetics, mechanisms, and consequences of diuron resistance have been reported (Jansen et al., 1993; Shigematsu et al., 1989). This may

provide an experimental system to test the effects of expression of the diuron-specific rFabs described in this paper.

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Supporting Information Available: Articles on cDNA sequence, three-dimensional structure, and properties of the recombinant diuron Fabs and previous publications on the derivation and use of the monoclonal antibodies from which the rFabs were cloned (50 pages). Ordering information is given on any current masthead page.

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