Cloning and Expression in *Escherichia coli* of an Anti-Cyclohexanedione Single-Chain Variable Antibody Fragment and Comparison to the Parent Monoclonal Antibody

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The single-chain immunoglobulin variable region genes of a murine anti-cyclohexanedione IgG-producing hybridoma were assembled and cloned into a bacterial expression vector. The single-chain antibody fragment (ScFv) was assembled from the variable regions of the γ heavy chain (IgG_1) and the κ light chain from the monoclonal antibody. The single chain was assembled by PCR using a $(Gly_4Ser)_3$ linker between the carboxyl end of the variable heavy chain and the amino end of the variable light chain. The ScFv fragment was expressed in *Escherichia coli*. An enzyme-linked immunosorbent assay was developed to examine the interaction of the recombinant ScFv with selected cyclohexanedione analogues. The cross-reactivity profile of the recombinant ScFv was found to be similar to the parent monoclonal antibody.

Keywords: *ELISA; ScFv; phage display; monoclonal; antibodies; cyclohexanedione; herbicides*

INTRODUCTION

Phage display technology offers a powerful means of generating antibodies more efficiently than conventional monoclonal system, with and without animal immunization, and can result in antibodies that have substantially improved specificity and affinity of antigen binding (Griffiths et al., 1993; Hoogenboom et al., 1992; Lerner et al., 1992; Marks et al.; 1991; O'Neil and Hoess, 1995; Winter and Milstein, 1991). Phage display directly couples the search and isolation of a biological property by linking the phenotypic property selection with the specific genomic coding sequence. In the filamentous phages *fd, f1*, and M13, the minor coat protein encoded by gene III (gene 3 protein or g3p) mediates the binding of phage to the F-pilus of *Escherichia coli* cells. During phage attachment, the N-terminus of g3p attaches to the host while the C-terminus remains anchored to the phage. Phage display technology is based on the observation that fusion of a foreign protein to the N-terminus of g3p does not affect normal phage assembly or infectivity. In fact, the foreign protein becomes "displayed" on the phage surface (Smith, 1985). This allows convenient screening and selection of the antibodies on the basis of biochemical interactions with a specific ligand or antigen (Hoogenboom et al., 1991; Kenan et al., 1994).

In this paper we report the successful cloning and expression in *E. coli* of the immunoglobulin variable region genes of an IgG-producing hybridoma produced against an anti-cyclohexanedione herbicide immunogen. The cross-reactivity profile of the ScFv against three cyclohexanedione analogues in an indirect enzyme-linked immunosorbent assay was compared to the parent monoclonal antibody.

MATERIALS AND METHODS

Cyclohexanedione−Protein Conjugates. *Immunogens.* The immunogen was produced by conjugating the cyclohexanedione analogue 1 (Figure 1) to bovine serum albumin (BSA) as described by Fleeker (1987) with the following modifications. A solution of equimolar amounts (95 mM) of analogue 1 (Figure 1), *N*-hydroxysuccinimide, and *N,N′*-dicyclohexylcarbodiimide in a total volume of 2.5 mL of dioxane was incubated in the dark, overnight at 22 °C. This mixture was centrifuged at 15000*g* in a Sorvall centrifuge for 10 min to remove the precipitate. The supernatant was dried under vacuum at 35 °C. The residue was resuspended in 3 mL of 0.15 M sodium borate buffer (pH 9.0) containing 200 mg of BSA. This mixture was incubated at 22 °C with stirring for 1 h and dialyzed against 4 × 1 L of phosphate buffered saline (PBS, 0.01 M phosphate, 0.15 M NaCl, pH 7.5) at 4 °C overnight.

Coating Conjugates. The coating conjugate for the indirect enzyme-linked immunosorbent assay was prepared by incubating, in the dark, 0.04 M analogue 1 (Figure 1), 0.11 M *N*-hydroxysuccinimide, and 0.26 M 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide in 400 μL of pyridine at 22 °C. After 1 h, 200 μL of this solution was added to 1 mL of 0.14 M NaCl containing 5 mg of ovalbumin (OVA). The mixture was incubated in the dark, at 22 °C for 1 h with slow stirring, followed by dialysis in PBS as described above.

Production of Monoclonal Antibodies. *Immunization.* Ten 8-week-old Balb/C mice were injected intraperitoneally with a 1:1 (v/v) mixture of 140 μg of immunogen dissolved in PBS (pH 7.4) and Freund's complete adjuvant in a total volume of 250 μL. Secondary immunizations were given 2 and 5 weeks after the initial immunization. The secondary immunizations consisted of a 1:1 (v/v) mixture of 70 μg of immunogen in PBS (pH 7.4) and Freund's incomplete adjuvant in a volume of 250 μL. Additional boosts (tertiary immunizations) were administered at 3-week intervals following the secondary immunization and consisted of 70 μg of immunogen in 250 μL of PBS (pH 7.4). Each week following the secondary and tertiary immunizations, mice were bled and the anti-cyclohexanedione sera titers determined using the indirect ELISA procedure as described by Deschamps et al. (1990). All mice immunized with the analogue 1-BSA conjugate tested positive for cyclohexanedione-specific antisera production. Mice possessing the highest specific antisera titers were given a final *intravenous* immunization consisting of 70 μg of immunogen in 250 μL of PBS and 3 days later sacrificed by cervical dislocation.

Hybridization and Fusion Product Screening. The methods described by Deschamps et al. (1990) were followed for hybridization. Ten days following hybridization, cell culture supernatants were screened for the presence of anti-cyclohexanedione-specific antibodies as described by Deschamps et al.

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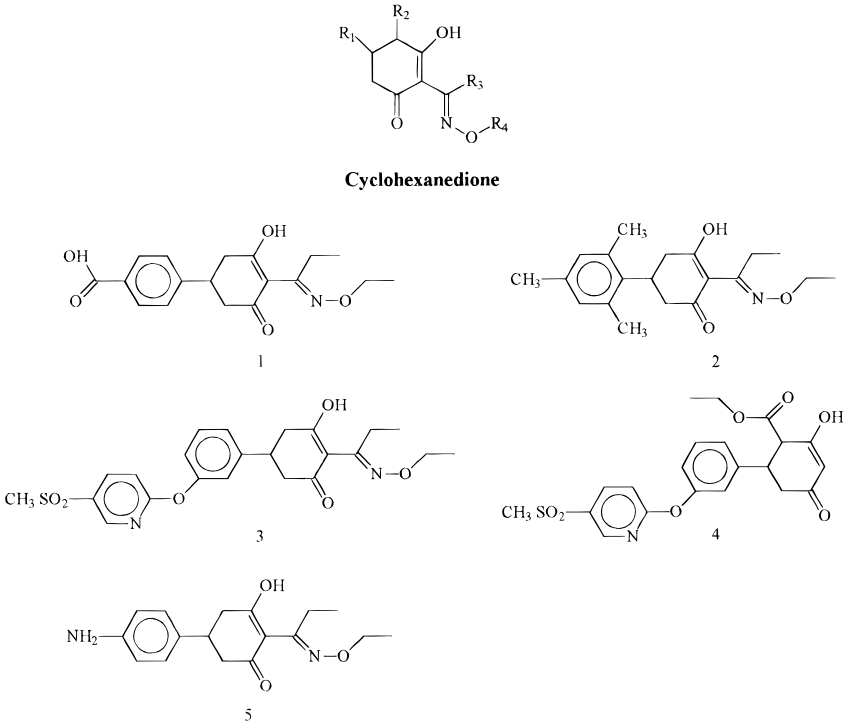

Figure 1. General structure of a cyclohexanedione herbicide and various herbicidally active and inactive cyclohexanedione analogues. Analogue 1 was conjugated to bovine serum albumin and ovalbumin and was used as the immunogen for the production of the parent monoclonal antibody and the ELISA coating conjugate (CCA), respectively. Analogue 1 was also conjugated to ω-(aminohexyl)-sepharose 4B and used to affinity purify the ScFv antibody fragment. Analogues 2 and 3 are active cyclohexanedione inhibitors of acetyl coenzyme-A carboxylase activity, whereas analogue 4 is an inactive inhibitor. Analogue 5 was used to make the photoaffinity label.

(1990) except that microplates were coated with the cyclohexanedione−OVA coating conjugate (CCA). After 10 days, the culture supernatants from the 24-well culture plates were assayed for binding to coating conjugate CCA. Competition between the immobilized coating conjugate CCA and selected cyclohexanedione analogues were conducted and revealed which cultures produced antibodies which interacted with the free cyclohexanedione structure. One antibody-producing culture designated as mAbA was used.

Limiting Dilution. Cultures producing cyclohexanedione-specific antibodies were selected for limiting dilution to achieve clonality of the hybridoma cells. The cell concentration was estimated by Trypan blue viability stain and was diluted to give a final concentration of 1 cell/100 μL of medium. This dilution (100 μL/well) was dispensed into 96-well microplates and incubated as described above. The wells were checked daily for the presence of a single colony. Once a colony was observed, the culture medium was assayed for the presence of anti-cyclohexanedione antibody as described above. Cells from colonies testing positive were transferred to 24-well culture plates, rescreened, and subjected to a second limiting dilution to ensure monoclonality. After a final assessment for the presence of anti-cyclohexanedione antibody, hybridoma cells were collected for the production of ascitic fluid in mice.

Production of Acites Fluid. Approximately 3×10^6 cells from selected hybridoma cell lines in 200 μL of PBS were injected intraperitoneally into Balb/C mice. These mice were treated 10 days earlier with 500 μL of pristane (Aldrich) intraperitoneally. Three weeks following injection of the hybridoma, the peritoneal cavity was distended and the ascites fluid collected.

Isotyping. The monoclonal antibody heavy and light chain isotypes were determined using the Pierce enzyme-linked immunosorbent assay isotyping kits using CCA as the antigen.

Cloning and Expression of Anti-Cyclohexanedione ScFv Fragments. General DNA and RNA manipulations were performed as described by Ausubel et al. (1987) and Sambrook et al. (1989). The recombinant anti-cyclohexanedione-specific ScFv antibody fragments were produced using the Recombinant Phage Antibody kit supplied by Pharmacia Biotech, Inc. (Baie d'Urfé, Québec, Canada). A detailed description of the rationale and protocol is provided in Jackson et al. (1992). Instructions and reagents supplied by the manufacturer were used, with some modifications as described below.

Messenger RNA Isolation and First-Strand cDNA Synthesis. Hybridoma cells producing the anti-cyclohexanedione-specific antibody were cultured in RPMI medium containing 10% (v/v) fetal calf serum and 100 mg/mL of gentamycin sulfate in a 5% (v/v) CO_2-humidified incubator. Cells were grown to a density of 1.5×10^5/mL and pelleted by centrifugation at 1000*g* in a Sorvall centrifuge. Cells were resuspended in 2 mL of TRIzol reagent, lysed by pipetting, and total RNA isolated according to the manufacturer's instructions (Gibco BRL). mRNA was purified by affinity chromatography using the Poly(A) Quik mRNA purification kit from Stratagene (La Jolla, CA). Purified mRNA was precipitated by ethanol, washed, and air-dried. The precipitate was suspended in 15 μL of diethyl pyrocarbonate (DEPC)-treated water. A 6-μL aliquot of the mRNA solution served as the template for first-strand cDNA synthesis as described by the manufacturer (Pharmacia).

Amplification of Immunoglobulin Variable Regions by Polymerase Chain Reaction (PCR). PCR amplification of both the immunoglobulin heavy (V_H) and light (V_L) chain variable region genes was performed using the cDNA template and oligonucleotide primers (provided by the manufacturer) complementary to the 5′ and 3′ coding and noncoding sequences of the immunoglobulin variable regions. PCR was carried out for 30 cycles using the high-fidelity *Pfu* DNA polymerase (Stratagene) under the following conditions: denaturation at 94 °C for 30 s; annealing at 55 °C for 1 min; and extension at 72 °C for 1 min. PCR products were visualized on a 1% (w/v) agarose gel stained with ethidium bromide.

Assembly of ScFv Antibody Fragments. The PCR amplified DNA products corresponding to the expected sizes of the V_H (340 bp) and V_L (320 bp) fragments were purified using low melting point agarose (Pharmacia). The putative V_H and V_L DNA were assembled into a single chain with a linker DNA by PCR. The assembly reaction contained equimolar concentrations of V_H and V_L, along with $(Gly_4Ser)_3$ linker primer and appropriate mixtures of dNTPs, PCR buffer, and *Pfu* DNA

polymerase (Jackson et al., 1992) in a final volume of 50 μL. PCR amplification of the linked ScFv antibody DNA was performed as described by the manufacturer, except the reaction volume was 100 μL. In this reaction, the primers also contained *Sfi*I and *Not*I restrction sites at their respective 5′ ends to facilitate directional cloning. The assembled ScFv DNA fragment was gel purified, ethanol precipitated, washed, and resuspended in sterile water.

Cloning and Expression of ScFv Antibody Fragments. The ScFv DNA was restricted with *Sfi*I and *Not*I and ligated into the phagemid pCANTAB 5E (Jackson et al., 1992; Pharmacia). The ligation product was transformed into competent *E. coli* TG1 cells. Transformed cells were plated onto a solid agar medium containing 2% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.05% (w/v) NaCl, 0.05% (w/v) $MgCl_2$, 0.05% (w/v) D-glucose, and 0.01% (w/v) ampicillin (SOBAG) and grown overnight at 30 °C. Ampicillin-resistant clones were identified and transferred individually to 125-mL Erlenmeyer flasks containing 10 mL of 1.7% (w/v) tryptone, 1% (w/v) yeast extract, 0.5% (w/v) NaCl, 2% (w/v) D-glucose. and 0.01% (w/v) ampicillin (2xYT-AG). Cultures were incubated overnight at 37 °C with shaking at 250 rpm. Cells were harvested by centrifugation at 13000*g* for 10 min using a Sorvall centrifuge. The phagemid DNA was isolated and restricted with *Sfi*I and *Not*I. Only clones with the correct 750-bp ScFv DNA insert were selected for further study.

Recombinant phages displaying the ScFv antibody fragment were produced by infecting transformed TG1 cells with the filamentous phage M13K07. The phages were isolated and used to infect the nonsuppresser *E. coli* HB2151 strain to produce soluble ScFv antibody fragments (Jackson et al., 1992). Transformed HB2151 clones were selected on SOBAG plates containing 0.01% (w/v) nalidixic acid. Transformed HB2151 clones were transferred to 5 mL of 3.5% (w/v) tryptone, 2.0% (w/v) yeast extract, and 0.5% NaCl (SB medium, pH 7.5) containing 2.0% (w/v) D-glucose and 0.01% (w/v) ampicillin. The cultures were incubated overnight at 30 °C with shaking at 250 rpm. Cells were harvested by centrifugation and the recombinant phagemid isolated and restricted with *Sfi*I and *Not*I. Only clones with the correct 750-bp fragment were used to produce soluble ScFv antibody fragments. Soluble ScFv fragments were produced with an E-tag peptide sequence fused to their carboxyl terminus to facilitate screening by an anti-E-tag antibody (Pharmacia).

Production of Recombinant Anti-Cyclohexanedione ScFv. *Expression of Anti-Cyclohexanedione ScFv.* A single colony of *E. coli* HB2151 harboring the recombinant phagemid was transferred to 30 mL of SOBAG medium containing 0.01% (w/v) nalidixic acid and incubated overnight at 30 °C in a 125-mL Erlenmeyer flask with shaking at 300 rpm. A 20-mL sample of the inoculum was transferred to 200 mL of SB medium containing 2.0% (w/v) D-glucose and 0.01% ampicillin in a 500-mL loosely capped baffled flask. The culture was incubated at 30 °C for 8 h as described above. The culture was centrifuged for 15 min at 1500*g* in a Sorvall refrigerated centrifuge. The cell pellet was washed in SB medium and centrifuged as described above. The resulting pellet was resuspended in 200 mL of SB medium containing 0.01% (w/v) ampicillin and isopropylthiogalactoside (IPTG) added to a final concentration of 5 mM. The culture was incubated overnight at 30 °C with shaking. Cells were harvested by centrifugation and the pellet washed in ice-cold PBS (pH 7.5) followed by centrifugation, after which the pellet was stored at −20 °C until analysis.

Preparation of Cell Extracts. The cell pellet was thawed and resuspended in sufficient ice-cold PBS (about 4−6 mL) to form a thin paste. About 1.5 g of alumina (Sigma Chemical Co.) was added, and cell extracts were prepared by breaking the cells by sonication (Webb and Lee, 1991). After sonication, cell debris and fragments were removed by centrifugation at 13000*g* for 10 min. The supernatant was further clarified by centrifugation at 30000*g* for 1 h (Lee et al., 1986).

Affinity Chromatography of the ScFv Antibody Fragments. The affinity matrix was prepared by coupling the cyclohexanedione analogue 1 (Figure 1; 2-((1-ethoxyimino)propyl)-5-(carboxyphenyl)-3-hydroxy-2-cyclohexen-1-one) to ω-(aminohexyl)-sepharose 4B with 1-ethyl-3-(3-(dimethylamino)propyl)-carbodiimide according to manufacturer's instructions (Sigma Chemical Co., St. Louis, MO). The affinity column (1 × 5 cm) was previously equilibrated with 100 mL of phosphate-buffered saline (PBS, 10 mM phosphate, 0.15 M NaCl, pH 7.5) at 0.5 mL/min, and 5-mL fractions were collected. The cell extract was diluted into 3 vol of PBS and loaded onto the column. The column was washed with 5 vol of PBS (30 mL) followed by 20 mL of 24 mM citric acid monohydrate and 47 mM sodium phosphate buffer (pH 5.0). The bound ScFv fragments were eluted with 30 mL of 0.1 M glycine−HCl buffer (pH 2.5). Eluted ScFv fragments were immediately neutralized with 1 mL of 1 M Tris-HCl buffer (pH 8.0). Protein concentration of each fraction was determined by the method of Bradford (1976) using bovine serum albumin as the standard. Fractions containing ScFv activity were pooled, concentrated, and washed with PBS on an Amicon ultrafiltration apparatus using a 10-kDa molecular weight cut off YM-10 membrane.

Indirect Enzyme-Linked Immunosorbent Assay (ELISA). The coating conjugates (1 ng/well) in PBS were passively absorbed to 96-well flat-bottom polystyrene microplates (Immunlon 4, Fisher Scientific Co.) at 4 °C overnight. Unbound coating conjugate was removed by washing with 0.05% (v/v) Tween 20 in PBS (PBST). Unoccupied sites were blocked with 200 μL/well of 0.1% (w/v) gelatin in PBS for 30 min at 22 °C. Serial dilutions of monoclonal antibody or ScFv fragments were prepared in PBS, and 100 μL/well of this solution was incubated with the bound coating conjugate for 1 h as described above. Unbound antibody or ScFv fragments were washed in PBST as described above. Goat anti-mouse IgG (1:5000 dilution in PBS) conjugated to horseradish peroxidase (Pierce) was incubated (100 μL/well) with plates containing the monoclonal antibody/coating conjugate complex for 1 h at 22 °C. The plates with ScFv fragments were incubated with an anti-E-tag monoclonal antibody diluted 1:2500 in PBST containing 0.1% (w/v) gelatin for 1 h at 22 °C. The ScFv plates were washed as above, and 100 μL/well of goat anti-mouse IgG conjugated to horseradish peroxidase was added and incubated for 1 h as previously described. All wells were washed with PBST, and 100 μL/well of substrate, 0.01% (w/v) urea/hydrogen peroxide (Sigma Chemical Co.), and 0.1% (w/v) 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) in a 24 mM citric acid monohydrate and 47 mM sodium phosphate buffer (pH 5.0) was added. The color reaction was terminated after 30 min of incubation at 22 °C by the addition of 100 μL/well of 0.5 M citric acid. Color development was quantified by measuring absorbance at 405 nm using a Bio-Rad Model 3550-UV microplate reader.

Inhibition Studies. Equal volumes of a known concentration of a cyclohexanedione analogue and a dilution of monoclonal antibody or ScFv antibody fragment were incubated for 30 min at 22 °C. The mixture was then incubated on plates containing the immobilized coating conjugate for 1 h. Once incubated with goat anti-mouse-horseradish peroxidase antibody conjugates and substrate, color development was inversely proportional to the original concentration of the cyclohexanedione analogue.

Synthesis of Photoaffinity Label. The photoaffinity label was synthesized by covalently linking (*N*-hydroxysuccinimidyl)-4-azidosalicylic acid (NHS-ASA) to the free primary amine of analogue 5 (Figure 1). NHS-ASA and 1,3,4,6-tetrachloro-3α,6α-diphenylglycouril (Iodo-gen) were purchased from Pierce (Rockford, IL). NHS-ASA and analogue 5 were dissloved in 500 μL of dimethyl sulfoxide to concentrations of 10 and 2.5 mM, respectively. NHS-ASA and analogue 5 in DMSO were added to 4.5 mL of tricine−KOH buffer at pH 8.3 (50 mM tricine−KOH, 10% (v/v) glycerol, 1 mM EDTA, 15 mM $NaHCO_3$, pH 8.3), and the emulsion was stirred for 1.5 h at 22 °C in the dark. The reaction was quenched with 275 μL of 1 M Tris. The photoaffinity label was iodinated using the Pierce Iodo-gen method according to the manufacturer's instructions. Protein was labeled by adding 25 μL of photoaffinity label solution to 250 μL of protein solution, incubating for 1.5 h at 22 °C in the dark, then subjecting the reaction vial to 10 high-intensity photo flashes. Photoaffinity labeled

proteins were separated by SDS-PAGE (12%) and visualized by autoradiography.

Analytical Techniques. *Gel Electrophoresis*. The purity of the ScFv antibody fragments was assessed using 12% (w/v) polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli (1970). SDS-PAGE was carried out at 150 V for 40−60 min in a Bio-Rad Mini Protein II Gel Electrophoresis apparatus. Proteins were stained with silver nitrate using the Bio-Rad Silver Staining kit prior to visualization.

Western Blot Analysis of Expressed ScFv Antibody Fragments. Protein samples were separated on 12% SDS-PAGE and transferred from the gels onto poly(vinylidene difluoride) (PVDF) membranes (wet with methanol) using the Bio-Rad Mini-Protean II TransBlot apparatus in the presence of 15.6 mM Tris and 120 mM glycine in 10% (v/v) methanol (transfer buffer) following instructions provided by the supplier. Transfer efficiency was monitored using broad-range prestained SDS-PAGE standards (Bio-Rad; control 75722). Following transfer, the membrane was blocked overnight in 100 mL of blocking buffer (PBST + 0.1% (w/v) gelatin). The membrane was washed three times in PBS followed by incubation in PBS for 5 min. The membrane was incubated for 2 h with anti-E-tag monoclonal antibody (1:2500, Pharmacia) diluted in blocking buffer, followed by three 5-min washes in PBS. The membrane was incubated with goat anti-mouse conjugated to horseradish peroxidase (Pierce) diluted 1:5000 in blocking buffer for 2 h and washed in PBS as described above. Color development was carried out in PBS containing 0.06% (w/v) chloronaphthol, 0.018% (v/v) hydrogen peroxide, and 10% (v/v) methanol for 30 min.

RESULTS AND DISCUSSION

Monoclonal Antibody Production. The cyclohexanedione analogue 1 (Figure 1) was used to produce both the immunogen and the ELISA coating conjugate. Analogue 1 (2-(1-(ethoxyimino)propyl)-5-(carboxyphenyl)-3-hydroxy-2-cyclohexen-1-one) is an active inhibitor of acetyl coenzyme-A carboxylase with IC_{50} values of 11.6 nM. An active inhibitor of acetyl coenzyme-A carboxylase was selected for producing the immunogen in order to present the cyclohexanedione pharmacophore to the immune system.

The cyclohexanedione pharmacophore centers around the cyclohexanedione ring (Figure 1) and includes the oxygen at position one, an oxyimino group with alkyl or alkenyl halide substituents at position 2, a hydroxyl group at position 3, and either an alkyl or aryl substituent at position 5. Cyclohexanediones possessing herbicidal activity are believed to mimic the transition state of acetyl coenzyme-A carboxylase during catalysis (Winkler et al., 1989). The actual role(s) of these groups in binding to the acetyl coenzyme-A carboxylase herbicide binding site is(are) unknown. However, it is thought that the groups at positions 1−3 may be involved in electrostatic interactions with specific amino acids at a common anion pocket on the enzyme. The size of the alkyl or alkenyl halide substituents at R_4 influences the activity of cyclohexanedione analogues. Generally ethyl and propyl substituents at R_4 are more active than methyl or hydrogen substituents, whereas more bulky alkyl susbstituents such as isopropyl and *tert*-butyl are less active. For cyclohexanedione analogues with alkyenyl halide substituents such as clethodim, the trans configuration is more active than cis. It has been shown that lipophilic substituents at position 5 facilitate binding to acetyl coenzyme-A carboxylase (Kobek and Lichtenthaler, 1990).

When conjugated to BSA, analogue 1 (Figure 1) was observed to elicit an immune response in all animals tested. All mice immunized with the immunogen were found to possess an anti-cyclohexanedione specific titer. Of the 10 mice initially immunized with the analogue 1-BSA conjugate, competitive ELISA studies indicated that five mice (M_{2A}, M_{3A}, M_{5A}, M_{6A}, and M_{8A}) produced antisera with high specificity for analogue 1. The first fusion attempt using mouse A_8 resulted in only one single stable anti-cyclohexanedione-specific hybridoma cell line designated mAbA.

Cloning and Expression of ScFv Antibody Fragments. Messenger RNA was isolated from anti-cyclohexanedione antibody producing hybridoma cells (mAbA), cDNA was synthesized, and PCR was performed using immunoglobulin V region-specific 5′ coding and 3′ noncoding oligonucleotide primers. Following amplification of the V_H and V_L chain region DNA, the predominant products were of the expected sizes of the V_H (340 bp) and V_L (320 bp) fragments. These were purified, assembled, cloned into pCANTAB5E expression vector, and used to transform TG1 cells. Only TG1 clones containing the expected 750-bp fragment of the recombinant ScFv fragment (38 of 67 transformed clones) were used to produce recombinant phages. These recombinant phages were used to infect *E. coli* HB2151 cells to produce soluble ScFv fragments. A total of 12 HB2151 clones were recovered from SOBAG plates containing 0.01% (w/v) nalidixic acid, and only two clones (HBCHD12 and HBCHD19) retained the correct 750-bp ScFv DNA fragment, as assessed by restriction of the isolated phagemid with *Sfi*I and *Not*I. The loss of ScFv DNA following transfection of *E. coli* HB2151 has been reported previously (Hoogenboom et al., 1991; Tyutyulkova and Paul, 1994). For instance, Tyutyulkova and Paul (1994) reported 56% of the *E. coli* HB2151 clones recovered following transfection with recombinant phage contained functional soluble antibody fragments to a vasoactive intestinal peptide.

Recombinant ScFv fragments were detected in the culture medium, periplasmic space, and cytoplasmic extracts of HBCHD12 cells by dot blot analysis and photoaffinity labeling experiments using an ^{125}I-labeled cyclohexanedione analogue (data not shown). Although detected in all cell fractions, the remainder of the study focused on ScFv preparations from the whole cell (periplasmic space and cytoplasm) because of ease of handling. Western blot analysis showed the affinity purified anti-cyclohexanedione-specific ScFv fragment was the major band at 29.3 ± 0.4 kDa (predicted M_r 30 000) (Figure 2, lane 3). The nature of the high-MW band at about 98 kDa is unknown. One possibility may be due to the presence of the ScFv:pIII fusion protein, since previous studies have shown that antibody:pIII fusion products have similar MWs. For instance, an anti-lysozyme ScFv:pIII fusion protein migrated as a 93-kDa protein on Western blot analysis (Breitling et al., 1991). Cell fractionation studies showed the ScFv:pIII fusion proteins are located in the cytoplasmic and membrane fractions and not in the culture medium and periplasmic extracts (Boeke and Model, 1982; Breitling et al., 1991). In our study, this high-MW band was not present on Western blot of proteins from periplasmic preparations (data not shown). The presence of other minor bands may be attributed to partial proteolysis of the fusion protein. Western blot analysis of the whole cell extract revealed the anti-E-tag monoclonal antibody cross-reacted with many proteins (Figure 2, lane 2). In another study, Breitling et al. (1991) also found the monoclonal antibody to the linker sequence cross-reacted with several proteins in the whole cell extract.

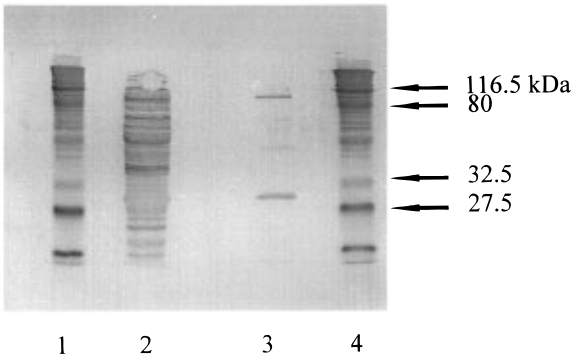

Figure 2. Western blot analysis of the anti-cyclohexanedione ScFv antibody fragment. Lanes 1 and 4 were loaded with the following prestained SDS-PAGE broad-range molecular weight protein standards: myosin (205 kDa), β-galactosidase (121 kDa), bovine serum albumin (86 kDa), ovalbumin (50.7 kDa), carbonic anhydrase (33.6 kDa), soybean trypsin inhibitor (27.8 kDa), lysozyme (19.4 kDa), and aprotein (7.4 kDa). Lane 2 was loaded with 7 μg of whole cell extract. Lane 3 was loaded with 5 μg of ScFv antibody fragment following enrichment on the cyclohexanedione affinity chromatography column.

The crude ScFv preparations in our study were not functional in the indirect competitive ELISA format. The lack of detectable activity in ELISA may be attributed to the low specific activity of the ScFv fragments in these crude preparations. However, once enriched on the cyclohexanedione affinity column, the recombinant ScFv fragments were suitable for use in the competitive ELISA format.

We recovered less than 20 TG1 transformants. Due to difficulties experienced in panning with phage-displayed ScFv's, these clones were used directly to transfect HB2151 cells, thereby circumventing the problems associated with panning. Difficulties with panning have been reported by Lee et al. (1995) while producing phage-display antibody fragments from the spleen cells of mice immunized with one of two mycotoxins (diacetoxyscirpenal and aflatoxin M1) rather than from a hybridoma cell line. First, they found that the nature of phage−ScFv binding to hapten/protein coated tissue culture flasks was poly-specific. There was considerable binding to both the protein and hapten. Second, regardless of the washing procedure used during the panning process, high nonspecific binding occurred. Third, when three different elution solutions (PBS, mycotoxin, or triethylamine mixed with Tris-HCl) were compared during panning, the use of mycotoxin to selectively elute high-affinity phage antibodies did not improve the panning procedure. Fourth, they found that phage ScFvs were unstable when stored. Finally, they had difficulty generating soluble ScFvs when they attempted to overcome some of the problems associated with the phage-displayed ScFvs. Lee et al. (1995) suggested that the problem may be related to generation of stable antibodies with reasonable affinity when panning the phage libraries. We also experienced many of these problems during the course of this research.

It is possible that more than one species of cloned ScFv sequences may be derived from the hybridoma cell line used in our study. Consequently, we characterized each of the 12 HB2151 clones for the correct size of DNA insert and functional soluble ScFv as determined by a binding assay. In our study, only one clone was found to have both binding activity and the correct insert size.

In ELISA, the cross-reactivity profiles of the ScFv fragment and parent monoclonal antibody with selected cyclohexanedione analogues were similar. The structures of the cyclohexanedione analogues tested for cross-reactivity are shown in Figure 1. Analogues 2 and 3 are active inhibitors of acetyl coenzyme-A carboxylase activity, whereas analogue 4 is not active. The concentrations of analogues 2 and 3 required to effect 50% inhibition of corn acetyl coenzyme-A carboxylase activity (IC_{50}) were 1 and 0.3 μM, respectively. The dose−response patterns for cyclohexanedione analogues 2−4 against the ScFv fragments and the parent monoclonal antibody are shown in Figure 3. The concentrations of analogues 2 and 3 required to inhibit 50% of the ScFv fragment binding (IC_{50}) to the coating conjugate were 183.9 ± 3.6 and 3.9 ± 0.8 nM, respectively. These are similar to the IC_{50} values found for analogue 2 (171.0

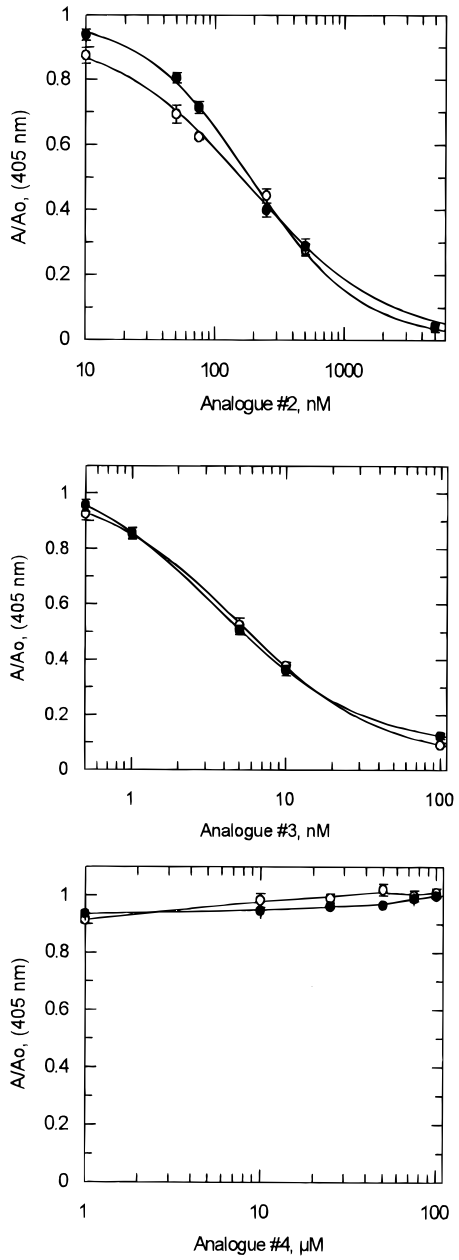

Figure 3. Effect of various concentrations of cyclohexanedione analogues 2−4 (Figure 1) on the binding of monoclonal antibody (○) and ScFv antibody fragments (●) to the cyclohexanedione−ovalbumin coating conjugate in an indirect competitive ELISA. Intra-assay and inter-assay coefficients of variation were less than 6 and 10%, respectively. Absorbance values of the standards and samples (A) were normalized by dividing by the absorbance values of the negative controls (A_0). Bars represent standard errors for three independent determinations.

± 8.4 nM) and analogue 3 (3.0 ± 0.5 nM) when tested for binding inhibition with the parent monoclonal antibody (Figure 3). The dose−response curve for analogue 4 indicates this inactive cyclohexanedione analogue at concentrations as high as 100 mM failed to inhibit binding of either parent monoclonal antibody or ScFv fragment to the coating conjugate.

The cyclohexanedione analogue cross-reactivity profiles and IC_{50} values indicate that the recombinant ScFv fragment has hapten specificity and affinity similar to that of the parent monoclonal antibody. These results suggest that the structural details of the parent monoclonal antibody antigen binding site are retained which permit high-affinity binding and discrimination between cyclohexanedione analogues in the recombinant ScFv fragment. It is noted that the most potent acetyl coenzyme-A carboxylase inhibitor, analogue 3, was also the most potent inhibitor of both ScFv fragment and monoclonal antibody binding to the coating conjugate. In addition, both the monoclonal antibody and ScFv fragment failed to cross-react with analogue 4, an inactive acetyl coenzyme-A carboxylase inhibitor.

Our results are similar to those of Cheadle et al. (1992) and Tang et al. (1995), who produced ScFv fragments with affinities and cross-reactivity profiles that closely resemble those of the parent monoclonal antibodies. Tang et al. (1995) produced ScFv antibody fragments from a digoxin-specific high-affinity monoclonal antibody. Similarly, He et al. (1995) reported that the three-domain antibody fragment ($V_{H/K}$) derived from a progesterone-specific monoclonal antibody also exhibited similar affinity and cross-reactivity profiles against related steroids. These studies suggest that ScFv fragments with properties similar to those of the native monoclonal antibodies can be produced against haptens. Some researchers have shown the ScFv fragments to exhibit reduced affinity when compared to the parent antibody (Bird et al., 1988; Colcher et al., 1990; Glockshuber et al., 1990; Huston et al., 1988; Lake et al., 1994; Malby et al., 1993; Wels et al., 1992). For the most part these ScFv fragments were produced against protein antigens such as lysozyme and insulin. In most cases, differences can be attributed to the additive impact on affinity measurements of two binding sites versus a single binding site in the antibodies and ScFv antibody fragments, respectively. For instance, Abraham et al. (1995) investigated the carcinoembryonic antigen binding kinetics of ScFv fragments and Fab fragments and compared them to the parent monoclonal antibody using surface plasmon resonance detection. The on-rates for all binding proteins were within the same order of magnitude (1×10^5 M^{-1} s^{-1}), whereas differences were observed in the off-rates. Both the monovalent ScFv and Fab fragments dissociated about 200 times faster than the parent monoclonal antibody.

In summary, we have obtained the functional expression and detection of a cyclohexanedione-specific recombinant single-chain Fv fragment in *E. coli* that displays cross-reactivity profiles similar to those of the parent monoclonal antibody. This expression system provides a means for further improvement of the binding affinity and specificity of the ScFv by protein-engineering methods. The production of recombinant ScFv fragments may prove useful for studying drug-target site interactions, e.g., by selecting antibody mimics of natural target sites.

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