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Molecular Cloning and Characterization of a Single-Chain Variable Fragment Antibody Specific for Benzoylecgonine Expressed in *Escherichia coli*

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Benzoylecgonine is a major metabolite of cocaine. We generated hybridoma cells (C1303) producing anti-benzoylecgonine monoclonal antibody (mAb) with a single-chain variable fragment (scFv) and an antigen-binding domain from the C1303 cells. Genes encoding an scFv antibody and constant region (Fc) were amplified from a cDNA library of C1303 cells using PCR. The two frameworks built for scFv and scFv-Fc consisted of HL [(heavy chain variable region, V_H) - linker - (light chain variable region, V_L)] and HL-Fc, respectively. A 45 base-pair-long sequence encoding $(Gly_4\text{-}Ser)_3$ was used as the linker, and the mouse IgG1 constant region sequence (225 amino acids) was used as the Fc domain. These two types of recombinant Abs were determined to be 750 bp in length (which corresponds to a 30 kDa protein) in the HL and 1,432 bp in length (which corresponds to a 65 kDa protein) in the HL-Fc, respectively. The parental Ab and HL-Fc affinities against benzoylecgonine were measured by ELISA and found to be nearly equal to the Ab concentration. We were also able to measure HL affinity using an agarose diffusion assay (Ouchterlony test). The affinity of the recombinant single-chain antibody against benzoylecgonine was sufficiently comparable to that of the parent antibodies to be used for the immunodetection of specific drug compounds or the detoxification of drug abusers by immunotherapy.

***Keywords*:** cocaine, benzoylecgonine, single-chain antibody, parental antibody, hybridoma, hinge region

Drug addiction is a disease that requires immediate treatment because most drug abusers cannot abstain from taking drugs when the drugs are readily available. New treatment options for this disease include the use of vaccines or drug-specific antibodies directed against drugs of abuse as a means of preventing relapse of drug-taking behavior. Drug vaccines induce drug-specific antibodies in the bloodstream, which bind to the drug of abuse and prevent it from entering the brain. Several studies demonstrated that immunization against cocaine can reduce drug intake and drug-seeking behavior in rats and mice (Carrera *et al*., 1995; Kantak *et al*., 2000; Carrera *et al*., 2001). Vaccines for cocaine and nicotine can generally offer long-term protection with minimal treatment compliance. Unlike the prevention of relapse of drug-taking behavior by the vaccine, high-dose treatment of drug-specific antibodies affects the rapid neutralization of toxic materials in the bloodstream. During the past decade, many groups have explored the feasibility of using drug-specific antibodies for the treatment of drug addiction. Heroin-specific antibodies successfully suppressed drug self-administration in monkeys (Bonese *et al*., 1974). Successful immunotherapy for drug abuse and overdose requires drug-specific antibodies with a high affinity and selectivity for target drug molecules. Passive immunization with mAbs has been examined for cocaine in preclinical models as like the studies using active immunization. The studies have focused on the ability of mAb to affect drug discrimination and relapse to drug use. In rats, passive immunization reduced reinforcing effects, and reinstatement of cocaine self-administration after low-dose cocaine (Carrera *et al*., 2000; Carrera *et al*., 2001). The recombinant of a humanized anti-cocaine mAb was reported with a modest K_D value for cocaine of about 200 nM (Redwan *et al*., 2003). This mAb was reengineered from a mouse anti-cocaine mAb, which has successfully reduced the reinstatement of cocaine use, a rat model of human relapse (Carrera *et al*., 2000).

In this study, benzoylecgonine antibody was selected as a model system for immunotherapy for drug abuse. Derived from the leaves of cocoa plant, cocaine is a potent central nervous system stimulant as well as a local anesthetic. The greatest psychological effects induced by cocaine include euphoria, confidence, and a sense of increased energy, accompanied by an increased heart rate, pupil dilation, fever, tremors, and sweating. The continued ingestion of cocaine could lead to tolerance and physiological dependency, which leads to its abuse. The main routes of cocaine use include smoking, intravenous, intranasal or oral administration, and it is excreted in the urine, primarily as benzoylecgonine, within in a short period of time. Benzoylecgonine has a biological half-life of 5~8 h, which is much longer than that of cocaine (0.5~1.5 h), and it can generally be detected within 12~72 h after cocaine use or exposure. The common feature of anti-cocaine antibody is its ability to recognize the phenyl ring and methylester group of cocaine molecules,

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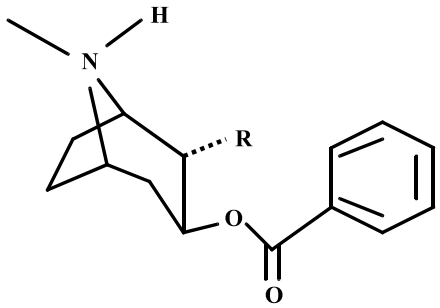
which can easily cross-react with major cocaine metabolites, such as benzoylecgonine (see Fig. 1), ecgonine, and ecgoninemethylester.

Any type of antibody to be used for immunotherapy should be humanized to avoid immune rejection in the human body. Except for intact humanized antibody, Fab or single-chain Fv recombinant antibodies are useful alternates for therapeutic approach, as they are known to have various advantages (Marasco, 1997; Cochet *et al.,* 1998; Marasco, 2001). These specifically engineered antibodies can bind a defined target epitope without any cross-reaction with other cellular antigens. In addition, they can be constructed to have a higher affinity and specificity to target molecules by modifying appropriate site sequences (Cohen, 2002).

In order to construct a recombinant antibody against a specific antigen, a gene encoding a variable domain of the antibody should be isolated from a cDNA library of an antibody-producing cell. Two different methods can be applied to obtain a specific gene that encodes a specific antibody. One is the phage library screening method, which allows for the selection of antibody fragments against a variety of antigens, such as viral proteins, membrane receptors, or cytokines (Griffiths *et al*., 1994; Vaughan *et al*., 1996). However, most of the antibodies selected by this method have a low or intermediate affinity to the target molecule, and therefore an extensive screening process is required in order to select a proper antibody with the desired affinity. Another approach is to derive scFvs or Fab fragments directly from mouse or rat hybridoma cells that produce target-specific antibodies. In this method, the isolated target gene can be expressed *E. coli* system, where the antibody affinity and specificity against target molecules can be easily done. Both scFv and Fab show similar properties to those of the parental antibody in terms of affinity and specificity.

ScFv is a single polypeptide chain composed of one heavy chain variable region (V_H) linked to one light chain variable region (V_L) through a flexible spacer, a repeated motif of 3× GGGGS (Huston *et al.*, 1991) or divalent form linkers (Kontermann and Müller, 1999). The V_H-linker-V_L sequence can be inverted without any loss of its binding activity.

In this study, C1303 hybridoma-producing anti-benzoylecgonine antibody was prepared in order to construct a recombinant single-chain benzoylecgonine antibody. The scFv gene was isolated from the cDNA of C1303 and expressed in *E. coli.* The recombinant single-chain antibody was characterized using several types of immunoassay.



R	Name
-COOCH3	Cocaine
-COOH	Benzoylecgonine

Fig. 1. Structure of cocaine and its major metabolite, benzoylecgonine.

Materials and Methods

Preparation of the benzoylecgonine monoclonal antibody producing hybridoma

In order to prepare monoclonal antibodies directed towards benzoylecgonine, hybridoma cells were prepared by conventional procedures, generally following the methods described by Kohler-Milstein (Kohler and Milstein, 1975). Four-week-old female Balb/c mice were immunized with benzoylecgonine-BTG conjugate (Immunetics Inc., USA) in order to obtain antibody-producing B cells from mouse spleen cells. Immunogen was prepared according to the following method: benzoylecgonine-BTG conjugate (1 mg/ml) was diluted in 250 μl of PBS and emulsified with an equal volume of complete Freund's adjuvant (Sigma, USA) for the primary injection and incomplete Freund's adjuvant for the boosting injection. Emulsified immunogen was injected into the peritoneal cavity of mice. After two booster injections were administered in two weeks interval the primary injection, 1 μg of benzoylecgonine-BTG conjugate in PBS was delivered to the immunized mouse by tail vein injection as a final boost. In the Kohler Milstein method, SP2/0-Ag-14 mouse myeloma cells are fused with antibody-producing B cells from immunized mouse spleen cells in order to obtain hybridoma cells. Briefly, spleen cells released from a dissected spleen are passed through a 100-μm Nylon cell strainer (Falcon, USA), collected and mixed with 10^7 cells of SP2/0-Ag-14 myeloma cells in a 50 ml Falcon tube. After centrifugation at 400×g for 10 min, the supernatant was carefully removed without disturbing the pellet. One milliliter of 50% polyethylene glycol 3500 (Hybrimax PEG 3500 from Sigma, USA) was added to the mixed cell pellet, drop by drop, with vigorous stirring for 1 min. Another milliliter of DMEM was added slowly for 1 min, and 10 milliliter was added over the next 1 min. The fused cells were collected by centrifugation for 5 min at 400×g and then carefully resuspended in 100 ml of selective HAT medium (DME supplemented with 20% FBS, antibiotics, and HAT) by swirling. Each 100-μl cell suspension was transferred into 96-well plates and incubated under an atmosphere of 5% CO_2.

About 2 weeks after the fusion, the culture supernatants were collected and screened by ELISA. Positive clones were expanded and frozen in a liquid nitrogen tank. To isolated single cell clones, the cells expanded from positive clones were cloned by limiting dilution.

Purification of monoclonal Abs

To obtain monoclonal antibodies, hybridoma cells (1×10^7) were injected into the peritoneal cavity of retired Balb/c mice primed with pristine (Sigma, USA) for 14 days. Ascites were collected from hybridoma cell-injected mice after 10~14 days. Affinity chromatography using a protein G column (Pharmacia, USA) was used for antibody purification from mouse ascites. Ascites were clarified by centrifugation for

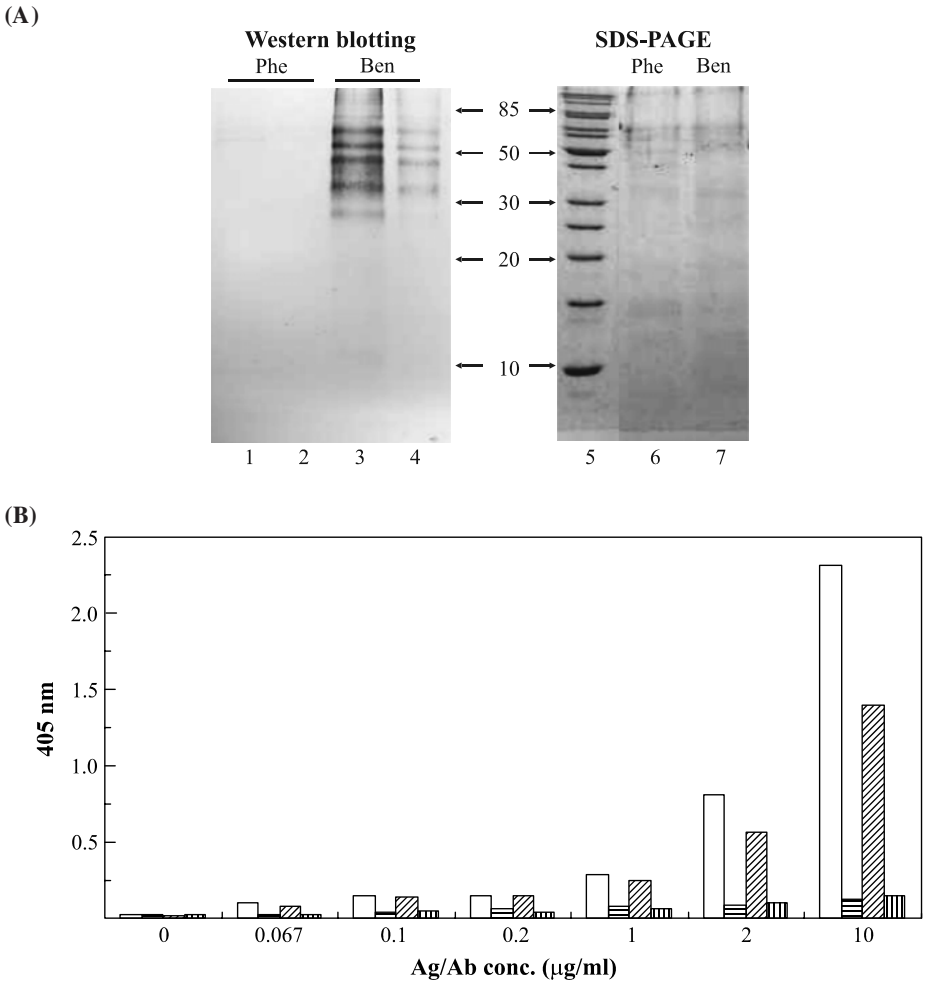


Fig. 2. (A) SDS-PAGE (right) and Western blot (left) of benzoylecgonine-BTG (Ben) and phencyclidine-BTG (Phe). Lane 1 and 2 contain 2 μg and 5 μg of Phe, respectively; lane 3 and 4 contain 5 μg and 2 μg of Ben, respectively; lane 5 contains a molecular weight marker; lane 6 and 7 contain 5 μg of Phe and Ben, respectively. (B) ELISA with mAb or antigen serial dilution. To confirm the Ag specificity, benzoylecgonine (□) and phencyclidine (▤) antigens were diluted, as shown along the bottom line of the figure, and reacted with the first Ab (benzoylecgonine mAb, 60 μg/ml). Conversely, benzoylecgonine (▨) and phencyclidine (▥) antigens were fixed to 2 μg/ml and tested at various concentrations of benzoylecgonine mAb (as shown in the Figure).

30 min at 15,000×g and passed through a 0.45-μm filter. Prior to apply to a protein G column (packed volume, 5 ml), the filtered ascites were diluted with four volume of 0.1 M sodium phosphate buffer (pH 7.5). Protein G column-bound antibodies were eluted with 0.1 M Glycine-HCl, pH 2.5. Each fractions (3 ml/tube) were collected by using peristaltic pump (flow rate 3 ml/min, EYELA, Japan), and neutralized immediately by the addition of 1 M Tris-base (90 μl) and dialyzed against PBS. Aliquots of the column fractions were electrophoresed for confirming the purity.

Cloning of a single-chain variable fragment from a C1303 hybridoma cell

Variable domain genes of the benzoylecgonine antibody were prepared from a cDNA library of hybridoma C1303 cell-producing monoclonal antibodies against benzoylecgonine. The C1303 cells were expanded to 10^8 cells in 5 bottles of 75 cm^2 culture flasks in order to purify the total RNA. Total RNA for the preparation of a cDNA library was extracted using TRI reagent according to the protocol suggested by the manufacturer (Molecular Research Center, Inc.). A cDNA library was constructed from extracted total RNA as follows. Five micrograms of total RNA mixed with oligo $(dT)_{12\text{-}15}$ primers, 25 mM $MgCl_2$, 10 mM dNTP, and 0.1 M DTT was transcribed by Superscript II reverse transcriptase (Life Technologies, USA) for 50 min at 42°C and the reaction was stopped by heating for 15 min at 70°C. Template RNA was removed by RNase H.

V_H and V_L gene fragments were amplified from the cDNA library by PCR using the following specific primers: V_H reverse primer; 5'-AGG TSM ARC TGC AGS AGT CWGG-3', V_H forward primer; 5'-TGA GGA GAC GGT GAC CGT GGT CCC TTG GCC CC-3'. V_L reverse primer; 5'-GAC ATT GAG CTC ACC CAG TCT CCA-3', V_L forward primer-1; 5'-CCG TTT GAT TTC CAG CTT GGT GCC-3', V_L forward primer-2; 5'-CCG TTT TAT TTC

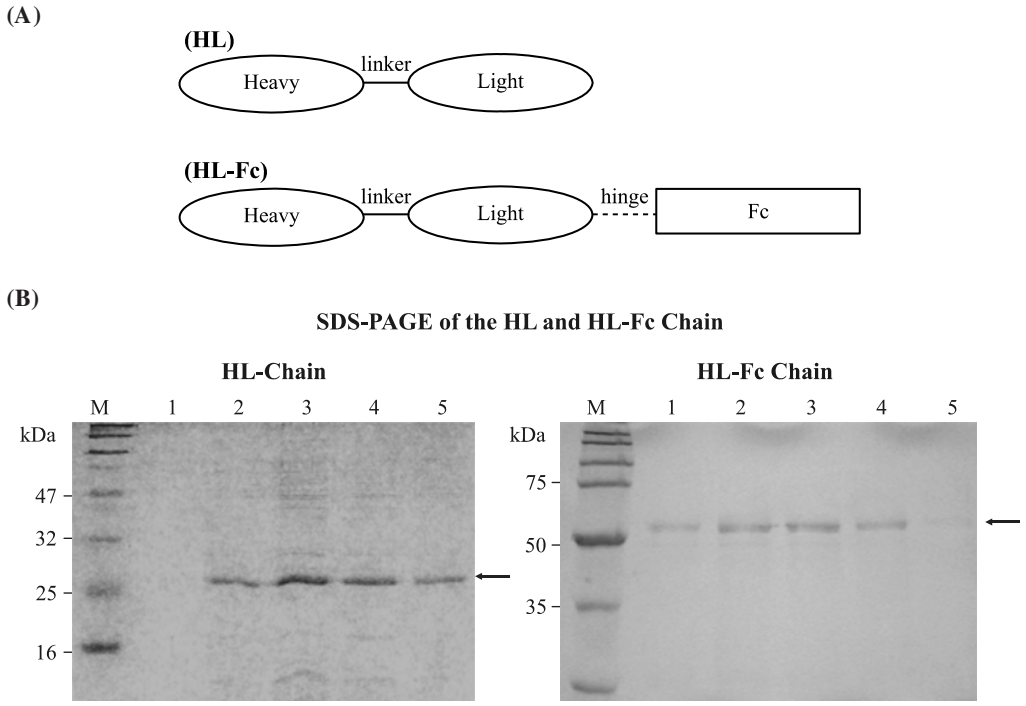


Fig. 3. (A) Scheme of recombinant single-chain Ab derived from hybridoma-producing benzoylecgonine monoclonal antibody. HL, heavy chain variable region is linked to the light chain variable region through a $(Gly_4Ser)_3$ linker; HL-Fc, HL is linked to the Fc region through the hinge region. (B) The recombinant HL (left) and HL-Fc (right) chain purified from *E. coli* was resolved by SDS-PAGE under reducing conditions and stained with Coomassie brilliant blue. The positions of protein size markers are shown on the left side. Lane numbers indicate the column fraction numbers.

CAG CTT GGT CCC-3', V_L forward primer-3; 5'-CCG TTT TAT TTC CAA CTT TGT CCC-3', V_L forward primer-4; 5'-CCG TTT CAG CTC CAG CTT GGT CCC-3'.

Hinge and Fc region gene fragments were amplified using the following specific primers: mIgG1 Hinge SfiNcoBack-5'; 5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC GTG CCC AGG GAT TGT GGT TGT AAG-3', mIgG1CH3BstNot For-3'; 5'-GAG TCA TTC TGC GGC CGC GGT GAC CTT ACC AGG AGA GTG GGA GAG GCT-3'.

The PCR reaction was carried out under the following conditions: 1 cycle for 5 min at 95°C, 30 cycles for 30 sec at 95°C, 30 sec at 55°C, and 1 min at 72°C.

Sewing of heavy-light chain genes

Cloned light and heavy chain fragments were amplified using linker contained primers and combined by using sewing primers that included restriction enzyme sites for cloning. The linker consisted of 45 base-pairs with $(Gly_4\text{-}Ser)_3$ sequences, and the total combined heavy-light chain spanned about 750 base pairs in length. Before heavy-light (HL) chain sewing, the heavy chain fragment was amplified by PCR using linker sequences (HL-4) and an enzyme sequence primer (HL-1), respectively. The light chain was amplified using linker sequences (HL-3), and the enzyme sequences contained the HL-2 primer. The amplified DNA fragments (H-linker and linker-L) were combined with the HL-1 and HL-2 (2-1, 2-2, 2-4, and 2-5) primer sets to construct the HL chain.

The HL-1 primers contained *Bam*HI, *Eco*RI, and *Sfi*I enzyme sites in the 5' terminus and the HL-2 primer contained a *Not*I site in the 3' terminus.

The primers used were as follows: HL-1; 5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG TSM ARC TGC AGS AGT CWG G-3', HL-2-1; 5'-GAG TCA TTC TGC GGC CGC CCG TTT GAT TTC CAG CTT GGT GCC-3', HL-2-2; 5'-GAG TCA TTC TGC GGC CGC CCG TTT TAT TTC CAG CTT GGT CCC-3', HL-2-4; 5'-GAG TCA TTC TGC GGC CGC CCG TTT TAT TTC CAA CTT TGT CCC-3', and HL-2-5; 5'-GAG TCA TTC TGC GGC CGC CCG TTT CAG CTC CAG CTT GGT CCC-3'.

PCR was performed for 1 cycle for 5 min at 95°C, 30 cycles for 30 sec at 95°C, 30 sec at 60°C, and 2 min at 72°C. The following primers were used to construct the scFv-hinge-Fc gene: mLC SfiNcoBack-5'; 5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC GAC ATT SAG CTS ACC CAG TCC-3' and mHCNcofor-3'; 5'-AGC TTC GAA TTC CAT GGT TGA GGA GAC GGT GAC CGT GGT-3'. The ScFv DNA was amplified using the primer above and digested with *Nco*I. The *Nco*I-digested fragment of ScFv was cloned into an expression vector that contained the murine hinge-Fc gene. Finally, the ScFv fused to the hinge-Fc gene was constructed as shown in Fig. 3A.

Construction and expression of the scFv antibody gene

Reamplified PCR products were digested with *Bam*HI and *Not*I and then resolved on 1.5% agarose to recover the scFv gene. DNA was extracted using a QIAGEN Kit (Germany).

The DNA fragment cloned into the multi-cloning sites of pET-28a-c cloning vector that carry an N-terminal His-Tag/thrombin/T7-Tag and C-terminal His-Tag sequences (Novagen, USA). Nucleotide sequence analysis was performed using the Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystem, USA) with an ABI 373 DNA sequencer. Recombinant plasmid was transformed into competent *E. coli* BL 21(DE3). Bacteria were grown with constant shaking in 2× YT broth (20 g tryptone, 10 g yeast extract, 10 g NaCl per 1 L) in the presence of kanamycin (50 μg/ml). For the induction of recombinant protein, 5 ml of cell suspension inoculate was added to 50 ml of 2× YT fresh media/250 ml flask, and then incubated until 0.6 (OD_{600}) at 37°C. The culture suspensions were further incubated for 1 h at 20°C in the presence of 1 mM IPTG with vigorous shaking (180 rpm). The bacteria pellet and media were harvested by centrifugation at 4°C, and the pellet was resuspended in 8 M urea (5 ml) and incubated overnight at 4°C. The media was concentrated with a speed vacuum to a 10-fold volume. $(His)_6$-tagged scFv were purified from the 8 M urea resuspension pellet and concentrated media by affinity chromatography on Ni-NTA agarose beads (QIAGEN). Bound molecules were eluted with 1 ml of 250 mM imidazole (in 50 mM sodiumphosphate, 300 mM sodium chloride, pH 8) and dialyzed against PBS (pH 7.4). The eluted proteins were stored at -70°C until further use. The eluted protein fractions were treated with reduced SDS-PAGE sample buffer and heated at 95°C for 5 min for application to 15% SDS-PAGE, which was carried out on a mini-protein electrophoresis apparatus (Bio-Rad, USA).

ELISA

A 96-well micro titer plate (Costar, USA) was coated with 50 μl of Benzoylecgonine-BTG conjugated antigens (1.0 μg/ml in PBSN), incubated overnight at 4°C and washed three times with de-ionized distilled water. The wells were blocked with 250 μl of borate buffered saline (BBS) containing 5% skim milk, 1 mM EDTA, 0.05% NaN_3, and 0.1% Tween 20 for 30 min at room temperature. After washing three times with de-ionized distilled water, 50 μl of Ab sample diluted with blocking buffer was added, and the mixture was incubated for 2 h at room temperature. After the well was washed three times with water, 50 μl of 1:1500 diluted goat anti-mouse IgG antibody conjugated with alkaline phosphatase (Sigma) was incubated for 2 h at room temperature. After the well was washed with water and blocking buffer, a 100 μl of 1 mg/ml (3 mM) p-nitrophenyl phosphate in 0.05 M Na_2CO_3, 0.05 mM $MgCl_2$ was added for detection. The reaction was stopped with 25 μl of 0.5 M NaOH, and the optical density was measured at 405 nm on a microtiter plate reader (Bio-Rad, USA).

Western blot and immunostrip assay

Benzoylecgonine-BTG conjugated antigens (1 μg) were resuspended in SDS-PAGE sample buffer, heated, and resolved on 12% SDS-PAGE. Gel-separated antigens were electrophoretically transferred to a PVDF membrane using a mini protein II transfer chamber (Bio-Rad). The membrane was blocked with blocking buffer (5% skim milk in PBS) overnight at 4°C. After washing with PBS containing 0.1% Tween 20 (PBS-T), diluted recombinant antibodies (60 μg/ml) in blocking buffer were incubated for 40 min at room temperature. The membrane was washed twice with PBS-T and incubated with goat anti-mouse IgG antibody conjugated with alkaline phosphatase (AP) for 2 h at room temperature. The membranes were washed five times with PBS-T and five times with distilled water and developed using 1 mg/ml (3 mM) *p*-nitrophenyl phosphate in 0.05 M Na_2CO_3, 0.05 mM $MgCl_2$.

To quickly confirm the binding activity of the recombinant antibody during the screening process, an immunochromatography assay was carried out by using a membrane strip, on which sheep anti-mouse IgG antibody was immobilized. Before applying the samples to the strip, the parental or recombinant antibody to be tested was mixed with benzoylecgonine-BTG-gold particle complexes to make a reaction sample. The strip was put in 100 μl of pre-mixed reaction sample in 96 wells, and it was allowed to incubate until the end of the membrane turned a reddish color.

To confirm the activity against non-denatured Ag, an Ouchterlony test was also adopted for the scFv binding assay with benzoylecgonine-BTG complexes. Recombinant scFv and antigen complex were placed into a 1% agarose well and incubated overnight at 37°C to allow for diffusions. The agarose plate was then stained with Coomassie blue and de-stained with 10% NaCl solution.

Results

Production of parental benzoylecgonine mAb

In order to construct a monoclonal cocaine antibody, Balb/C mice were immunized with a benzoylecgonine-BTG conjugate as described in the 'Materials and Methods' section. After 3 or 4 boosting injections, the mice were sacrificed to isolate immunized spleen cells. The spleen cells were fused with myeloma cells in order to construct hybridoma-producing anti-benzoylecgonine antibody. Thirty-one positive clones were selected from the three independent fusion experiments by primary screening using ELISA and immunochromatography analysis. Some of the clones were eliminated because their binding activity to free benzoylecgonine molecules was lower than BTG-conjugated benzoylecgonine or because they completely lost their ability to produce mAb during expansion for secondary screening. Finally, we chose C1303 among 6 hybridoma clones selected from 31 primary screened clones for further studies, which are described in this article.

In the Western-blot analysis, the antibody produced by C1303 can specifically bind to the benzoylecgonine-BTG conjugate, and the benzoylecgonine-BTG-conjugate showed multiple bands with molecular weights in the range of 30 to 60 kDa on SDS-PAGE (Fig. 2A). The benzoylecgonine-BTG-conjugate has an estimated molecular mass of 36 kDa, and about 10 molecules of benzoylecgonine arebound to one molecule of BTG.

However, the same amount of phencyclidine-BTG (PCP) did not produce a band with benzoylecgonine mAb, as shown in Fig. 2A. A similar observation in benzoylecgonine specificity was detected by two pairs of ELISA (Fig. 2B). One is that the parental benzoylecgonine monoclonal antibody was

serially diluted with PBS buffer, and the other is that benzoylecgonine-BTG complexes were serially diluted with PBS in order to confirm the binding specificity, as shown in Fig. 2B. These data have also shown specific binding between benzoylecgonine and benzoylecgonine antibody. A BTG-phencyclidine, which served as a negative control, showed no binding to the benzoylecgonine antibody, indicating that our benzoylecgonine monoclonal antibody is specific for benzoylecgonine alone and not BTG or phencyclidine.

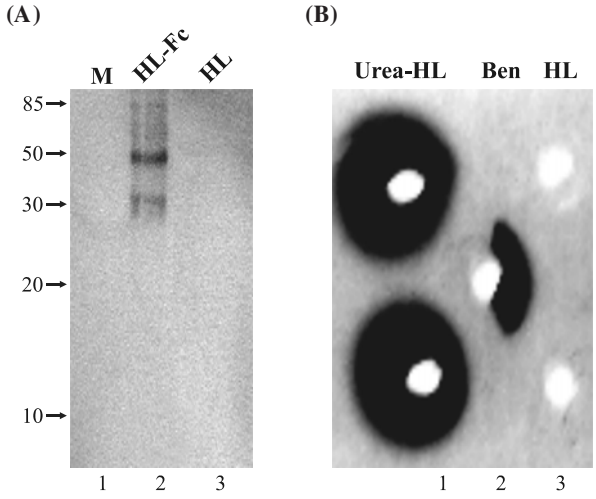


Fig. 4. (A) Western blot analysis of the benzoylecgonine recombinant antibodies. Benzoylecgonine-BTG Ag loaded onSDS-PAGE and transferred to the PVDF membrane. Recombinant HL (for negative control) and HL-Fc were used for the primary antibody. A goat anti-mouse IgG was used as the secondary antibody. Lane 1, molecular weight; lane 2, HL-Fc; lane 3, HL. (B) The expressed HL proteins were prepared from *E. coli* using urea and Ni-column, and loaded onto a 1% agarose well in order to allow for Ab-Ag complex formation. Urea-HL refers to the supernatant of 8 M urea treatment that was used to solubilize the insoluble protein, and HL refers to the protein eluted from Ni-NTA agarose beads. Ben refers to the benzoylecgonine - BTG coupled complexes as an antigen.

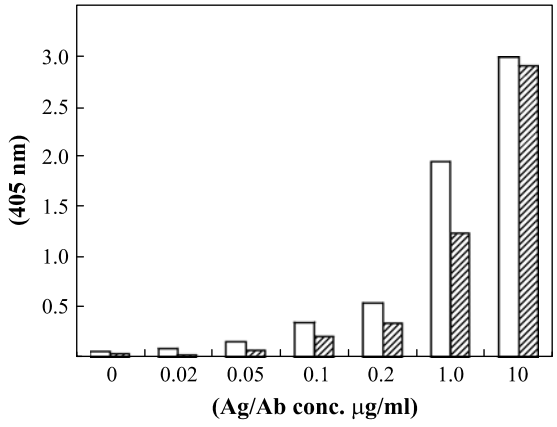


Fig. 5. ELISA of parental mAb and recombinant HL-Fc Ab. Benzoylecgonine-BTG Ag was adjusted to 2 μg/ml with PBS and coated onto 96-well microtiter plates. Recombinant HL-Fc (▨ and parental Ab (□ were diluted with PBS from 20 ng/ml to 10 μg/ml and used as the primary Ab. Goat anti-mouse IgG antibody conjugated with alkaline phosphatase was incubated for 2 h at room temperature. After reaction with substrate, the reactants were measured at 405 nm. The Y-axis indicates the observed substrate reaction color.

The generation of a recombinant single-chain variable fragment

The generation of HL from hybridoma relies on the amplification, assembly, and cloning of the V_H and V_L regions (Fig. 3A). Their expression as a single polypeptide chain was induced using prokaryotic vectors. For further analysis, we tried to attach the Hinge and Fc sequences to the linked the HL chain (Fig. 3A). The cloned V_H-V_L-Fc (HL-Fc) single-chain fragment was also expressed using the pET-28 expression vector in BL21 (Fig. 3B). The correct sizes of HL-Fc DNA fragments were amplified using specific primers, as described in the 'Materials and Methods' section. From the DNA sequence analysis, we confirm that cloned recombinants HL-Fc protein was comprised of mouse Hinge and Fc sequences (Fig. 3 and 6).

The HL fragment was 750 base pairs in length (which corresponds to a 30 kDa protein) and included 45 base-pair-long linker sequences, $(Gly_4\ Ser)_3$. The Fc portion of antibody attached to the HL chain was 1,432 base pairs in length (which corresponds to a 65 kDa protein). As shown in Fig. 3B, single band with molecular weights of 30 and 65 kDa were detected in the HL and HL-Fc fractions eluted from the Ni-NTA column, respectively.

The binding activities of the recombinant Abs were tested using a variety of methods, including the Ouchterlony assay and Western blotting.

Binding activity of recombinant antibody

Immunoassay of benzoylecgonine recombinant antibodies was evaluated by Western blot and an Ouchterlony assay. The Hinge and Fc portion attached to a recombinant single-chain Ab have shown binding activity against benzoylecgonine in the Western blot analysis, as shown Fig. 4A. HL chain was used for negative control because it did not contain the Fc portion (Fig. 4A, lane 3). While the HL chain activity could be measured using an agarose diffusion assay (Fig. 4B), the HL-benzoylecgonine-BTG complexes formed were not sharpened since the benzoylecgonine-BTG complex (Ben, hole 2) is not a uniform complex, as shown in Fig. 2A. As shown in Fig. 4B, the HL protein (hole 3) was purified using an Ni-NTA column and urea-HL samples (hole 1) containing 8 M urea. Urea was needed for purification of HL protein from inclusion body during Ni-NTA purification procedures (Fig. 4B).

We also confirmed this finding in a strip test (data not shown), which showed the same result as that shown in Fig. 4A. Parental benzoylecgonine antibody and HL-Fc were bound to a membrane that was linearized with sheep anti-mouse IgG. In these experiments, Fc-defective recombinant scFv (HL) was also used for negative control. These results indicated that the Ag binding site of the recombinant single chain was specific to benzoylecgonine.

To compare the parental Ab and HL-Fc affinities for Ag, the purified proteins were diluted to the same concentrations

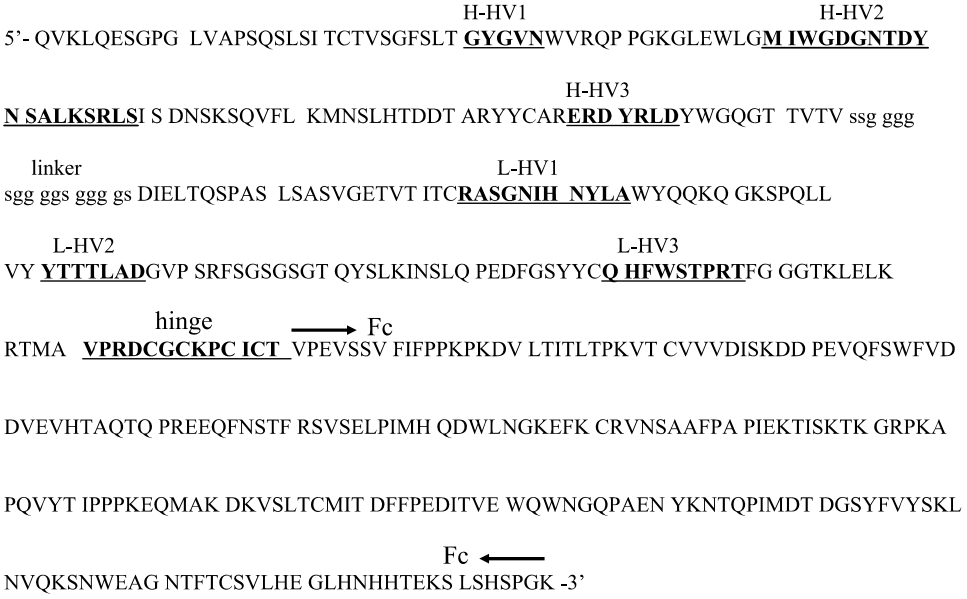


Fig. 6. The deduced amino acid sequences of the variable regions of recombinant heavy and light chain Abs (HL). Hypervariable regions (HV) are shown in bold and lined. Hinge and Fc sequences are indicated by underlined and arrowed letters, respectively.

so that the binding assay could be performed using ELISA (Fig. 5). As shown in Fig. 5, HL-Fc binding affinity towards the benzoylecgonine-BTG complexes reached 70% of that of the parental Ab up to a concentration of 1 μg/ml.

Sequences analysis

Recombinant HL and HL-Fc DNA was prepared for DNA sequences analysis. Sequencing analysis showed that the V_H, D, and J_H of benzoylecgonine Ab were derived from IGV_H2, D_H2, and J_H2, respectively, and the Vk and Jk of C1303 were derived from IGK12 and Jk5, respectively.

The translated amino acid sequences are shown in Fig. 6. The heavy chain of benzoylecgonine Ab belongs to mouse heavy chain subgroup IB, whereas the light chain belongs to the k chain subgroup V.

Discussion

When a hybridoma is used as an mRNA source for the construction of an scFv antibody, it is most desirable to use a cell line that produces a high-affinity antibody for the target hapten or antigen. This study describes the successful isolation and cloning of heavy and light chain Abs from benzoylecgonine-specific hybridoma C1303, their expression as an scFv protein, and their binding activities for the conjugate antigen. It has a potential advantage for binding to the defined target with minimal or no cross-reaction with other antigens, which may be useful in diagnosis.

Immunotherapies have specificities of the lack of addiction liability, minimal side effects, and long-lasting protection against drug use. It can also be coupled with other anti-addiction materials and improve behavioral therapies. More improved antigen design and antibody engineering will become highly specific and rapidly developed treatments for both existing and future addictions.

In this study, using several types of immunological assay, the C1303 scFv antibody was found to be reactive with benzoylecgonine Ag. It is noteworthy that our benzoylecgonine is one of only a few non-protein organic compounds reported in the literature (Schreiber *et al*., 1980).

After several immunizations with benzoylecgonine-BTG, the benzoylecgonine antibody was detected in the anti-sera of the immunized mouse. We also confirmed that the scFv engineered from anti-benzoylecgonine mAb (C1303) bound to benzoylecgonine just as the parental antibody. For the expression of cloned scFv, a bacterial leader peptide *pel*B (Lei *et al*., 1987) was attached in-frame to the N terminus of the recombinant antibody for periplasmic exportation from a reducing environment of the cytoplasm. Because each variable domain contains a disulfide bond and the oxidation of cysteine thiols into disulfides normally occurs in an oxidizing compartment, such as the periplasm, to form a stable fold in *E. coli* cells.

The V_H and V_L domains of benzoylecgonine scFv were connected with a linker $(Gly_4Ser)_3$ because Gly residues would confer the necessary flexibility, while Ser would provide some solubility (Huston *et al*., 1988; Pantoliano *et al*., 1991; Turner *et al*., 1997). A linker of an scFv antibody usually must be more than 12 amino acid residues long in order to exhibit similar reactivity to the parent antibody (Skerra *et al*., 1991; Kortt *et al*., 1994).

The binding activity of both the parent and scFv antibodies were determined for various concentrations of benzoylecgonine-BTG complex by ELISA. These antibodies showed similar specificities with each other.

The binding activity of the HL Ab was investigated using an Ouchterlony assay. In this experiment, classical sharp bands were not observed with the benzoylecgonine-BTG complex because the benzoylecgonine-BTG complex is not uniform, but is rather composed of aggregated complexes,

as shown in Fig. 2A.

The binding activity of the hinge and Fc portion attached to an HL chain was compared with the parental mAb using ELISA. The Fc attached to the scFv will be dimerized in *E. coli* with a disulfide bond, and it should bind to Ag with the same affinity as the parental Ab. We tried to obtain this result by using non-reduced polyacrylamide gel electrophoresis, but the obvious protein band could not be detected on the polyacrylamide gel with smeared or extremely faint patterns. The binding of separated V_H and V_L fragments via hapten antigen was reported with other anti-hapten antibodies (Suzuki *et al*., 2000; Arndt *et al*., 2001; Aburatani *et al*., 2003).

The recombinant heavy and light chain sequences revealed that the V_H, D, and J_H of the benzoylecgonine Ab were derived from IGV_H2, D_H2, and J_H2, respectively, and the Vk and Jk of C1303 were derived from IGK12 and Jk5, respectively.

This study is a useful example of the development of a recombinant antibody for a conjugate antigen, and we believe it can serve as a model for the development and construction of novel antibodies for the immunodetection of chemical drugs.

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