Urol Res (1996) 24:273–277
© Springer-Verlag 1996

ORIGINAL PAPER

L. Z. Yu · S. Xiao · H. L. Huang · Z. Gu · F. L. Gu
Y. L. Guo

Construction and expression of single-chain Fv antibody against human bladder carcinoma

Received: 13 June 1995 / Accepted: 9 February 1996

Abstract We designed two sets of oligonucleotide primers to amplify the immunoglobulin heavy- and light-chain variable-region genes from genomic DNA by polymerase chain reaction (PCR). The genomic DNA was extracted from hybridoma BDI-1 cells, which secreted a monoclonal antibody (mAb) against human bladder carcinoma. The primers contained special restriction sites that allowed the variable-region genes to be easily cloned for sequencing and expression. The recombinants were sequenced by Sanger's method. It was proved that the full lengths of the V_H and V_K genes were 366 and 324 bp, respectively. Compared with other published sequences, the V_H gene was a member of mouse heavy-chain V_H subgroup II and originated from the rearrangement of V_H, *Dsp*2.2 and J_{H4}. The V_K gene was V_K subgroup IV and from V_K and J_{K4}. The V_H and V_K genes was inserted expression vector pWAI80. By inducement, the ScFv antibodies were expressed and secreted from *Escherichia coli*. Binding activities against the bladder carcinoma cells were detected. We suggest that ScFv antibody recognized the antigen specifically.

Key words Single-chain Fv antibody · Expression · Polymerase chain reaction · Hybridoma · Bladder carcinoma · ELISA

Introduction

Bladder carcinoma is the commonest urological malignancy in China. It is frequency multifocal, and has a high recurrence rate even following apparently successful treatment with standard measures, such as ultimate radical cystectomy, chemotherapy and radiotherapy [13]. For the early detection and treatment of bladder carcinoma, one new modality worth considering is the use of monoclonal antibodies (mAbs) [1]. During the last decade we have produced and used mAbs for immunoimaging and immunotherapy [18–20]. In clinical practice, we have found several problems with mAbs. One major problem associated with their repeated use is the production of human antimouse antibodies (HAMAs), which may combine with the administered mAbs to reduce the effective dose able to reach the tumor site and in some cases produce allergic reactions in patients [18]. Ideally, human antibodies would be used. However, these have been proven difficult to make by the conventional method [6, 8, 15].

Recently, protein engineering has been used to convert mouse mAb into "human" mAb by joining the entire immunoglobulin variable (V) domains from mouse to human constant (C) domains [11] or by transplanting the complementarity-determining regions (CDRs) of the mouse mAbs into human antibodies [12]. One of these advances has been the development of recombinant single-chain antigen-binding proteins [11]. These are composed of a variable light-chain amino acid (V_L, V_K or V_γ) sequence of an immunoglobulin tethered to a variable heavy-chain (V_H) sequence by a designed peptide that links the carboxyl terminus of the V_L to the amino terminus of the V_H or vice versa. ScFv (Single-chain Fv) antibodies are expected to have several advantages in clinical application, one of which is that the ScFv antibody is much smaller than the original mAb, being therefore less immunogenic and may penetrate the microcirculation surrounding the solid tumor; it is also easily produced in *E. coli* [2, 4, 5, 17]. In this paper, we report on the construction and expression of ScFv antibody against bladder carcinoma.

L. Z. Yu (✉) · F. L. Gu · Y. L. Guo
Institute of Urology BMU, Beijing Medical University, Beijing, P.R. of China, 100034

S. Xiao · H. L. Huang · Z. Gu
Institute of Genetis, Academia Sinica, Beijing, P.R. of China 100101

Material and methods

Production and characterization of mAb

BDI-1 is a murine hybridoma established by ourselves, which produces a mAb (γ1/K) against bladder carcinoma. It was generated by the immunization of BALB/c mice with BIU-87 bladder carcinoma cell line, which was established in 1987 in our laboratory. Indirect immunofluorescence assay and ABC enzyme-linked immunoabsorbent assay (ELISA) immunohistochemical staining demonstrated that the BDI-1 mAb had a strong binding reaction with bladder carcinoma tissue, BIU-87 and E-J bladder carcinoma cell line, but that there was no reaction with normal bladder tissue, other human normal tissue and most fetal tissues. mAb BDI-1 recognizes a 114-kDa glycoprotein. Its detailed generation, characterization and reactivation have been described elsewhere [10].

Extraction of genomic DNA from hybridoma cells [14]

Hybridoma cell pellets were resuspended in a small volume of phosphate-buffered solution (PBS) and lysed in a volume of 0.5% sodium dodecyl sulfate (SDS), 100 mM NaCl, 100 mM TRIS-HCl (pH 8.0) and 100 mM ethylenediaminetetraacetate (EDTA) (pH 8.0), to give approximately 5×10^7 cells/ml. Protein was then digested first using RNase A at a concentration of 0.1 mg/ml for 1 h at 37 °C and followed by proteinase K at 0.25 mg/ml overnight at 55 °C. Next day the viscous solution was poured into a collodion bag and dialyzed 3 times against 4 L 10 mM TRIS-HCl (pH 8.0) and 20 mM EDTA (pH 8.0) until the OD_{270} of the dialysate was less than 0.05. The absorbance of the DNA was measured at 260 nm and 280 nm.

PCR amplification

PCR amplification was performed for both immunoglobulin heavy- and light-chain variable-region genes using DNA as template. We designed two sets of PCR primer for the mouse variable-region genes by computer analyses of published sequences of antibody gene. The primers for amplification incorporated restriction endonuclease sites for forced cloning. PCR was performed according to the standard protocols using *Taq* thermostable DNA polymerase, 30 cycles by denaturation for 1 min at 94 °C, annealing for 1 min at 55 °C and extension for 2 min at 72 °C. PCR products were visualized on an 8% polyacrylamide gel stained with ethidium bromide.

Cloning and sequencing

Amplified DNA was ligated into a sequencing vector pUC19 predigested with the blunt end restriction endonuclease *Hin*CII. Ligation was transformed into the fresh competent cells DH5α. Transformants containing insert were identified by white clone (plus insert) versus blue clone (minus insert). To ensure that white colony contained immunoglobulin variable region the plasmids were then digested with *Xba*I and *Eco*RI (V_K), *Xho*I and *Spe*I (V_H). If the plasmids contained an insert, they were subjected to DNA sequence analysis to further ensure that the inserts were anti-bladder carcinoma immunoglobulin variable-region genes. Double-stranded sequencing was performed using the T_7 sequencing Kit (Phamacia).

Construction and expression of single-chain Fv antibody

The PCR products in pUC19 were digested with *Xho*I/*Spe*I (V_H) and *Eco*RI/*Xba*I (V_L) and ligated with the vector pWAI80 (Fig. 1). The ligation product was transformed into *E. coli* XL1-blue and screened the recombinant with *Amp*. The recombinants carrying V_H and

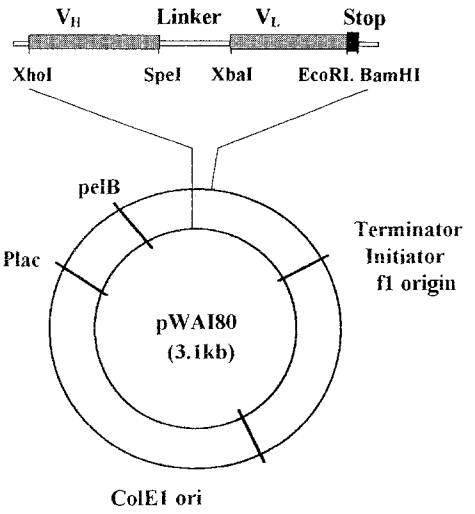

Fig. 1 Schematic drawing of the expression vector pWAI80. For antibody expression the vector contains a *pel*B signal sequence which directs ScFv and is released into the bacterial periplasm and suspernatant

V_L were identified by enzyme digestion. For the expression of ScFv, a single colony of recombinant was inoculated into 30 ml Super Broth [3% Bacto Trypton, 2% Bacto Yeast Extract, 1% (*N*-2-morpholino)propane sulfonic acid (MOPS), pH 7.0] containing 20 mM $MgCl_2$ and 0.05 mg/ml *Amp*, grown at 37 °C for 6 h ($OD_{600} = 0.2$) and induced by adding isopropyl thiogalactoside (IPTG) to the final concentration of 1 mM and continued to incubate overnight at 30 °C. The cells were recovered by centrifugation for 15 min at 1500 g. The cell pellet was resuspended in 1 ml PBS (0.8% NaCl, 0.02% KCl, 0.144% Na_2HPO_4, 0.024% KH_2PO_4, pH 7.4) and lysed by freezing in a dry ice-ethanol bath for 5 min followed by thawing at 37 °C in a water bath. This process was repeated 4 times. Pellet cell debris was centrifuged at 8000 g in an Eppendorf centrifuge. The supernatant was transfered to a fresh tube and used for ELISA.

Analysis of antigen-binding activities by ELISA

Four groups were used: (1) positive control: added BDI-1 only, (2) negative control: added noninduced lysate (NIL) only, (3) NIL + BDI-1 and (4) Lysate + BDI-1. The bladder carcinoma cells (BIU-87 or *E* – *J*, 10^6 cell/ml), 0.1 ml/well, were added to the wells of the ELISA plate, three wells/group. The supernatants were discarded after being centrifuged for 10 min at 400 *g*. The cells were washed twice with PBS. The fixing solution (0.5% glutaraldehyde), 0.1 ml/well, was pipetted for 3 min at room temperature and then discarded. The blocking solution (3% bovine serum albumin), 0.2 ml/well, was added and incubated for 3 h at 37 °C. In this step, different solution was added to each group (PBS, noninduced lysate, lysate) and incubated for 1 h at 37 °C. After washing the secondary antibody was added and incubated as above. Finally, added the third antibody (peroxidase-sheep anti-mouse IgG conjugate) and incubated for 0.5 h at 37 °C, washed, and then added substrate. The different OD values were shown on the Bio-Rad EIA Reader at 492 nm.

Results

Specificity of mAb

In order to prove the specificity of mAb, the bladder tumor cells were detected by indirect immunofluores-

cence. Second, immunohistochemical staining (avidin-biotin peroxidase method) of bladder carcinoma tissue demonstrated the extraluminar presence of the BDI-1 epitope. Third, radioimmunoscintigraphy of nude mice bearing bladder carcinoma cell xenografts and patients was conducted. Overall results showed both a high sensitivity and specificity of immmunoimaging for both the primer and recurrent tumor (Fig. 1).

PCR products

Total DNA was isolated from hybridoma cells. OD_{260}/OD_{280} was 1.9. Primers employed in this study were as follows:

V_L back: 5′ CCTCTAGAGAAATTGTTCTCACCCA-GTCTCC 3′ *Xba*I

V_L forward: 5′ CCGAATTCTTTTAGTTCCAACTTT-GTCCCCG 3′ *EcoR*I

V_H back: 5′ GGCTCGAGCAGGTGCAGCTGGTG-GAGTCTGG 3′ *Xho*I

V_H forward: 5′ GGACTAGTTGAGGAGACGGTGA-CTGAGGTTC 3′ *Spe*I

The major PCR products obtained after ethidium bromide staining are shown in Fig. 2. Their sizes are about 340 and 380 bp. Amplified DNA fragments encoding variable regions were selected by size in agarose and subcloned in pUC19 vector, thus allowing sequencing of both strands of the fragments.

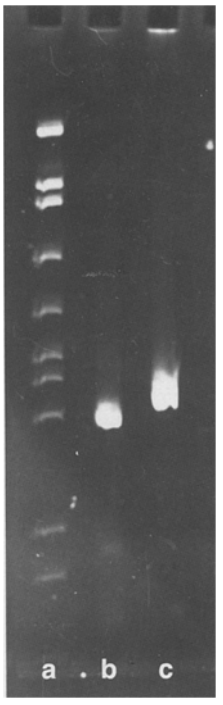

Fig. 3 PAGE (8%) of PCR products. **a** Length of molecular weight marker is 2645, 1605, 1198, 676, 517, 460, 396, 350 bp. **b** Amplified V_K gene by PCR. **c** Amplified V_H gene by PCR

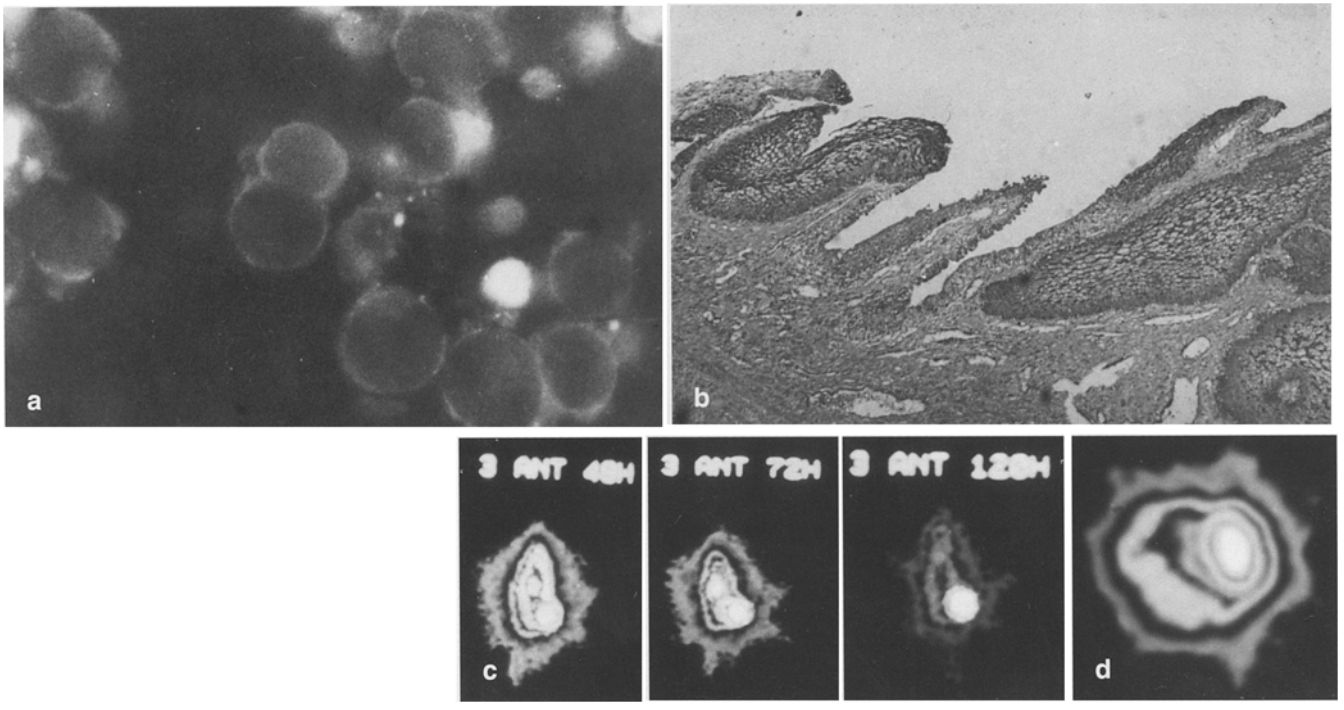

Fig. 2a–d Specificity of mAb against bladder carcinoma. **a** Tumor cells detected by indirect immunofluorescence, **b** immunohistochemical staining of bladder carcinoma tissue, **c** radioimmunoimaging of nude mice bearing bladder carcinoma cell xenografts at 48, 72, 120 h, **d** human primary bladder tumor detected by immunoscintigraphy after instillation with ^{131}I-mAb

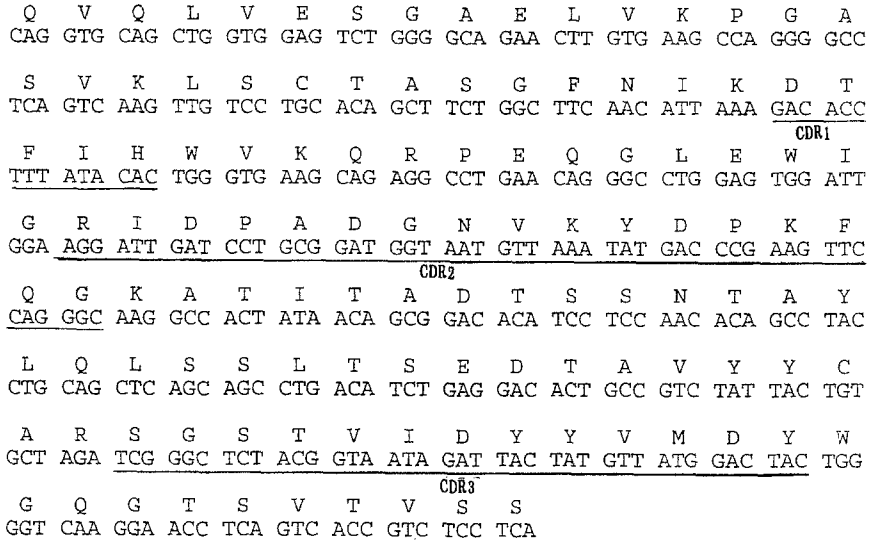

Fig. 4 Nucleotide sequence and deduced amino acid sequence of V_H gene of BDI-1.CDRs are underlined

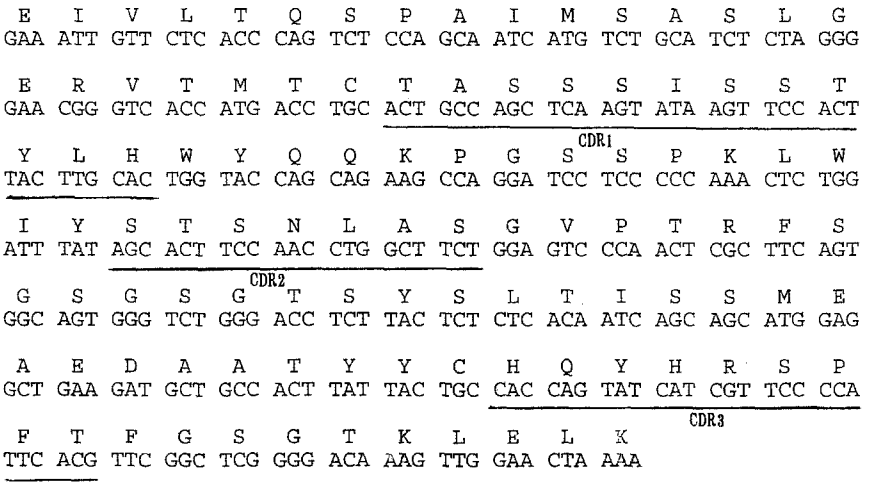

Fig. 5 Nucleotide sequence and deduced amino acid sequence of V_K gene of BDI-1. CDRs are underlined

Sequence analysis of variable regions

As shown in Figs. 3 and 4, a full length of the V_H gene was 366 bp and coded for 122 amino acids. The V_K region was 324 bp and 108 amino acids. They have been cloned successfully. Compared with other published sequences [7], the V_H gene is a member of the mouse heavy-chain V_H subgroup I and originates from rearrangement of V_H, *Dsp*2.0 and J_{H4}. The V_K gene was V_K subgroup IV and originated from V_K and J_{K4}. In addition, for proving the identity between the PCR products from genomic DNA and cDNA, cDNA was prepared (cDNA Synthesis Kit, Boehringer Mannheim) by reverse transcription from RNA (RNA isolation, Gibco BRL Kit) of the BDI-1 hybridoma cells, and amplified by the PCR procedure described above. The results showed that the sequence of PCR products of cDNA was the same as that of genomic DNA by sequencing.

Immunological activity assay

Because the ScFv antibody lacks the Fc fragment, the antigen-binding activity cannot be detected by usual ELISA, so we used the "immunological competing inhibition" method [3, 9]. The result (Table 1) showed that the lysate of induced clone 3 could inhibit 83% of the antigen-binding activity of original mAb BDI-1,

Table 1 Immunological competing inhibition (*NIL* noninduced lysate). Inhibition% = $(OD_n - OD_I)/OD_n \times 100\%$ (*n*: NIL + BDI-1, *I*: Lysate + BDI-1)

Groups	OD_{492}	Inhibition (%)
BDI-1 (positive control)	0.862	
NIL (negative control)	0.000	
NIL + BDI-1	0.715	
Lysate + BDI-1	0.121	83

and that the ScFv antibody in lysate could bind specifically to the epitope of bladder carcinoma cells.

Discussion

PCR is now widely used for cloning genes. Generallly, many laboratories use cDNA reverse-transcribed from RNA as template for PCR amplification. In this study we amplified variable-region genes from total DNA extracted with hybridoma cells by PCR technology. Because the immunoglobulin genes of hybridoma cells were rearranged, the V_H and V_K genes did not contain introns and could be amplified from total DNA directly. We consider that this method is feasible without RNA Isolation Kits and the cDNA Synthesis. Ward et al. [16] drew similar conclusions. By sequencing and by the fact that the sequences were consisted with the sequences of PCR product from cCNA it was proved that the V_H and V_K genes of mAb against bladder carcinoma were successfully amplified from total DNA of hybridoma BDI-1. The pWAI80 vector constructed by ourselves has multiple cloning sites for V_H and V_K genes and a linker between the genes, so the expressed product would be a single-chain Fv antibody. As the result of competing inhibition by ELISA, the ScFv antibody was specifically bound to the epitope of bladder carcinoma cells. The vector mainly secreted antibody into the periplasmic space of bacteria, although it contains the *pel*B signal sequence. Only a small quantity of ScFv protein was secreted into the medium, which was not enough to be detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The above results lead us to believe that the ScFv antibody against bladder carcinoma was successfully constructed and expressed. We expect that the antibody will be used for immunodiagnosis and immunotherapy of urological tumors as soon as possible.

Acknowledgement This work was supported by the Grant of Chinese National Education Commission.

References

1. Bamias A, Keane P, Krausz T, Williams G, Epenetos AA (1991) Intravesical administration of radiolabeled antitumor monoclonal antibody in bladder carcinoma. Cancer Res 51:724
2. Bird RE, Hardman KL, Jacobson JW, Johnson S, Kaufman BM, Lee SM, Lee T, Pope SH, Riorsan GS, Whitlow M (1988) Single-chain antigen binding protein. Science 242:423
3. Carl AK, Borrebaeck (1992) Expression and purification of antibody fragments using *E. coli* as a host. In: Antibody engineering. WH Freeman, New York, p 121
4. Carter P, Presta L, Gorman CM, Radway JBB, Henner D, Wong WLT, Rowland AM, Kotts C, Carver ME, Shepard HM (1992) Humanization of an anti-p185^{HER2}antibody for human therapy. Proc Natl Acad Sci USA 89:4285
5. Casey JL, Keep PA, Chester KA, Robson L, Hawkins RE, Begent RHJ (1995) Purification of bacterial expressed single chain Fv antibodies for clinical application using metal chelate chromatography. J Immuno Methods 179:105
6. Chatenpoud L, Baudrihaye MF, Chkoff N, Kreis H, Goldstein G, Bach JF (1989) Restriction of the human in vivo immune response against the mouse monoclonal antibody OKT3. J Immunol 137:830
7. Kabat EA, Wu TT, Perry HM, Gottesman KS, Foeller C (1991) Sequences of proteins of immunological interest, vol II, 5th edn. Public Health Service National Institute of Health, USA, pp 1250, 1350
8. Jams K, Bell GT (1987) Human monoclonal antibody production. J Immuno Methods 100:5
9. Johnson S, Bird RE (1991) Construction of single-chain Fv of monoclonal antibodies and their production in *Escherichia coli.* Methods Enzymol 203:88
10. Ma AH, Yu LZ, Xie SS, Gu FL, Long ZZ (1990) The production and characterization of a monoclonal antibody against human bladder carcinoma. Chinese J Urol 11:195
11. Morrison SL, Johnson MJ, Herzenberg LA, Oi VT (1984) Chimeric human antibody molecules: mouse antigen-binding domains with human constant region domains. Proc Natl Acad Sci USA 81:6851
12. Riechmann L, Clark M, Waldmann H, Winter G (1988) Reshaping human antibodies for therapy. Nature 322:323
13. Russel PJ, Plomly J, Raghavan D (1993) The use of monoclonal antibodies for the diagnosis and therapy of bladder cancer. Br J Urol 71:121
14. Sambrook J, Friutsch EF, Maniatis T (1989) Isolation of high-molecular-weight DNA from mammalian cells. In: Molecular cloning (a laboratory manual, 2nd edn.). Cold Spring Harbor Laboratory Press, pp 9, 14
15. Shawler DL, Bartholomew RM, Smith LM, Dillman RO (1985) Human immune response to multiple injections of murine monoclonal IgG. J Immunol 135:1530
16. Ward ES, Gussow D, Grifiths AD, Jones PT, Winter G (1989) Binding activities of a repertoire of single immunoglobulin variable domains secreted from *Escherichia coli.* Nature 341:544
17. Winter G, Harris WJ (1993) Humanized antibodies. Immunol Today 14:243
18. Yu LZ, Xie SS, Zhou Z, Na YQ, Yong Z, Gu FL (1991) Specific killing effect of monoclonal antibody BDI-1 conjugated adriamycin on bladder tumor. Chinese J Urol 12:243
19. Yu LZ, Gu FL, Zhang CL (1993) Radioimmunoimaging diagnosis of human bladder carcinoma. Scand J Urol Nephrol (Suppl) 157:13
20. Yu LZ, Gu FL, Han TS, Na YQ, Zhang ZL, Zhu CL, Pan ZY, Zhou C, Xie SS, Long ZZ (1993) Radioimmunoimaging diagnosis of bladder tumor with the intravesical administration of labelled monoclonal antibody. Chinese J Urol 14:252