New human single chain anti-idiotypic antibody against benzo[a]pyrene

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Abstract

The naïve library from the lymphocytes of healthy humans was screened by murine single-stranded idiotypic antibodies against benzo[a]pyrene (pSh). The phage clone which contained of anti-idiotypic antibody against benzo[a]pyrene, designated as A4, was chosen for further work because of highly specific to pSh. The available protein databases were searched. The A4 amino acid sequence was unique and 76% identical to a sequence in antibody against interferon γ. The A4 protein was expressed in bacteria and purified by two different methods: His-tagged A4 and CBD-fusion A4. Both the A4 bound to pSh and also to the human single chain idiotypic antibody against the benzo[a]pyrene (T72) by ELISA. The Kd values of A4 for pSh and T72 were very close: 4.44 × 10^-7 M and 5.71 × 10^-7 M, respectively. A4 was a competitor with benzo[a]pyrene for binding sites of both idiotypic pSh and T72 in competitive ELISA. Thus, A4 was a high affinity anti-idiotypic against benzo[a]pyrene which recognised pSh and T72 active sites.

Key words: anti-idiotypic antibody, benzo[a]pyrene, immunology, phage display, polycyclic aromatic hydrocarbons, single-chain antibody.

Introduction

The anti-idiotypic antibodies (Ab2) contain an internal immunological image of the antigen according to the concept Jerne N.K. [1, 2]. It was suggested that Ab2 be used for immunisation rather than antigen in the case of pathogenic antigen. Eichmann K. and Rajewsky K. 1975 [3] was one of the first works in this field, in which guinea pigs were immunised by Ab2 against Streptococcus. The animals acquired of resistance against bacteria after immunisation. Nowadays it has been proposed that Ab2 be used as therapeutic antibodies [4-9]. Ab2 were used against tumour-associated antigens in mice model experiments for protective and therapeutic anti-cancer immunity [10-14]. Ab2 were suggested for the treatment of cancer in humans [15-17], including lung cancer [18-20].

The preliminary immunisation by the murine monoclonal Ab2 against benzo[a]pyrene (BP) slowed tumour growth induced by BP in animals [21]. Ab1 against BP and related Ab2 were found in the blood serums of healthy volunteers and patients with lung cancer [22]. However, the functions of Ab1 and Ab2 against chemical carcinogens in human cancer pathogenesis still remain poorly understood. It was assumed that Ab1 and Ab2 were able to prevent the emergence of tumours and enhance the carcinogenic effects depending on the concentrations or combination of Ab1 and Ab2 [23]. The recombinant Ab2 against chemical carcinogens should be used to continue the research in this field. Ab2 could be applicable as a source of an antigen in human serum Ab1 detection because Ab2 contains an internal immunological antigen image. Also, purified Ab2 could be used as a standard for the development of quantitative immunoassays for measuring Ab2 in human serum.

Material and methods

Chemicals and reagents

Suppressor E. coli strain TG1 (K12, D(lac-pro), supE, thi, hisD5/F' traD36, proA+B+, lacIq, lacZDM15); non-suppressor E. coli strain HB2151 (K12, ara, D(lac-pro), thi/F' proA+B+, lacIq, lacZDM15); E. coli strain XL10-Gold (Stratagene, La Jolla, CA, USA) (end, A1, glnV44, recA1, thi-1, gyra96, relA1, lac, Hte, Δ(m-

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were obtained from Sibenzymes (Novosibirsk, Russia). The restriction enzymes in M13 bacteriophage [24]; pTT10 [kindly provided to us ICBFM SB RAS Novosibirsk]. The resulting enzymes were obtained from Sibenzymes (Novosibirsk, Russia).

pSh and T72 purification

pSh was mouse idiotype scFvs against BP. T72 was human idiotype scFvs against BP. Both scFvs purifications were processed as indicated in [25] and [26], respectively. pSh and T72 have CBD in each molecule. DNA of pSh and T72 were cloned into plasmid pTT10 [25]. So, amorphous cellulose was used for both protein purifications.

Synthesis of BP-BSA conjugate

BP-BSA was synthesised by covalent coupling of hapten aldehyde group to the BSA amino groups [27].

Biopanning

The selection was performed by phage display as has been described previously [24]. Three rounds of biopanning were performed using pSh. The microtiter plate was coated with 50 ml pSh (50 ng/ml) or CBD (50 ng/ml), as negative control, in PBS for one hour at 37°C. The plate was then blocked by adding 100 ml of blocking solution PBS containing 2% BSA and 0.05% Tween 20 to each well and incubated for one hour at 37°C with shaking. The bacteriophage particles of sampled M13 (containing scFv genes inside and expressing scFvs as part of the surface of the phage protein pIII) were added. The microtiter plate was incubated for one hour at 37°C with shaking. After washing, absorbed bacteriophage particles were eluted by triethylamine. When individual bacteriophage clones (48 clones) were analysed the bacteriophage particles sorption to CBD as negative control was extrapolated.

DNA sequencing and analysis

scFv DNA from TG1 bacterial clones was isolated by BioSilica columns (Novosibirsk, Russia). The DNA was sequenced using the primers LMB3 and pHEN-SEQ SEQ [28] and a sequencing kit BigDyeTerminator v3.1 Cycling Sequencing Kit (Applied Biosystems, Foster City, CA). The sequencing was performed in the inter-institutional centre of DNA analysis of the Siberian Branch of the Russian Academy of Sciences and using equipment of the Core Centre ‘Genomic Technologies, Proteomics and Cell Biology’ in the All-Russia Research Institute for Agricultural Microbiology.

T4 purification

The purification of His-tagged A4 was performed using Ni²⁺ resin as described in [26]. The resulting DNA from A4 of M13 phage encoding scFv against pSh was transformed into E. coli HB2151 strain for protein expression. Two hundred and fifty ml of LB containing ampicillin (150 mg/ml) were inoculated with 500 ml of an overnight culture of transformed E. coli and cells were grown at 37°C with vigorous shaking until an absorbance of 0.6–1 OD at 600 nm was attained. The induction of protein synthesis was induced by the addition of 1 mM isopropyl-beta-D-thiogalactopyranoside (Helicon, Novosibirsk, Russia). Overnight after induction, the bacterial cells were harvested by centrifugation and suspended in 6 ml of sonication buffer (PBS containing 100 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (Sigma, St. Louis, MO, USA). The disrupting of the bacterial cells and the chromosomal DNA was with four 30-s cycles at 70 watts in Vibra-cell Sonic power sonicator (Sonics, Newtown, CT, USA). Insoluble cellular membranes were removed by centrifugation at 25,000 × g for 15 min at 4°C. His-tagged A4 was in the supernatant and was purified by adsorption onto Ni²⁺ resin (Lab Instruments, Moscow, Russia) and elution with elution buffer (250 mM imidazole, pH 6.0), followed by dialysis against a buffer solution (400 mM Tris, pH 8.0, 500 mM NaCl, 1 mM EDTA) for 4 hours at 0°C. The protein refolding occurred during dialysis. Quality control for protein folding was tested by binding of antibodies to antigens. Such preparation of the His-tagged A4 was ~90% pure as assessed by SDS-PAGE. Concentration of purified protein was determined using the BCA Protein Assay (Thermo Fisher Scientific Waltham, Massachusetts, USA) and by spectrophotometry at 280 nm.

The A4 DNA was subcloned into the Ncol and BamHI sites of the pTT10 vector. In this case of CBD-fusion A4, the protein was prepared as described in [25].

SDS-PAGE

Gel electrophoresis was performed according to the method of Laemmli in 12.5% acrylamide gels. Proteins were detected in the gels by staining with 0.04% Coomassie G-250 (Bio-Rad Laboratory, Hercules, CA, USA).

Direct ELISA

Noncompetitive direct immunoassay was based on the specific binding of A4 to pSh or T72 [29]. The 96-well microtiter plates were coated with His-tagged A4 at 100 ng/ml at 37°C for one hour. After each step the plates were incubated at 37°C on a shaker for one hour. After each incubation step the plates were washed 3 times with PBS containing 0.05% Tween 20. After His-tagged A4 coating the plates were blocked by adding to each well 250 µl of blocking solution (PBS containing 0.5% BSA and 0.05% Tween 20). The pSh (from 0.8 ng/ml to 22 ng/ml) or T72 (from 0.2 to 12 ng/ml) were added. The rabbit anti-CBD Ab (1 : 1000) and then horseradish peroxidase-labelled an-
ti-rabbit conjugates of mouse Ab (1 : 5000) were added to the wells successively. The binding was detected by adding of tetramethylbenzidine. The production of coloured reaction was stopped by 2N HCl. The optical density was performed using wavelength of 450 nm on plate reader iMark (Bio-Rad Laboratory, Hercules, CA, USA).

In the case of CBD-fusion A4 the direct ELISA was the same except for the details. The 96-well microtiter plates were coated with CBD-fusion A4. Then after blocking pSh or T72 were added. Mouse anti-cMyc Ab (1 : 1000), which bound pSh or T72 and horseradish peroxidase-labelled anti-mouse conjugates of rabbit Ab (1 : 5000) were used.

**Competitive ELISA**

The competitive ELISA was evaluated as non-competitive ELISA (described above), except of the 96-well microtiter plates were coated with 100 ml of conjugate BP-BSA at 2 ng/ml for overnight at 6°C. The pSh or T72 (4 and 15 ng/ml, respectively, corresponding to half of the maximum saturation concentration – IC<sub>50</sub>) were added in

| Table 1. Analysis of the nucleotide sequences encoding V-, D-, and J-segments of heavy (A) and light (B) chains of A4. The comparison of the DNA sequences of A4 to germline segments |
| # | Germline segments | % identity | FR/CDR | Mutations | Amino acid residues replacements |
| --- | --- | --- | --- | --- | --- |
| A |  |  |  |  |  |
|  | IGHV1-69*13 | 93.75% | FR | 10 | a2>g, Q1>R (+ - -)  
g3>a, Q1>R (+ - -)  
t5>g, V2>G (- - -)  
g6ot, V2>G (- - -)  
c7>g, Q5>E (+ + -)  
c16>g, Q6>E (+ + -)  
t49>g, S17>A (- + -)  
g54>a |
|  |  |  |  |  |  |
|  | CDR 6 1 | g82>t, G28>Y (+ - -)  
g83>a, G28>Y (+ - -)  
g104>g, S35>T (+ + +)  
a106>g, S36>G (+ + -)  
t111>c  
g112>t, A38>Y (- - -)  
c113>a, A38>Y (- - -) |
|  | IGHV6*02 | 93.10% |  |  |  |
|  |  |  |  |  |  |
|  | IGHJ6*02 | 93.10% |  |  |  |
|  |  |  |  |  |  |
| B |  |  |  |  |  |
|  | IGKV3-20*01 | 95.04% | FR | 3 1 | a80>g, Q27>R (+ - -)  
g85>a, V29>I (+ + +)  
g110>c, S37>T (+ + +)  
g153>t, R51>S (- - -)  
t158>c, L53>P (- - -)  
a164>t, Y55>F (+ - -)  
g167>c, G56>A (- + -)  
g169>a, A57>T (- - -)  
g135>a  
g320>b, Y107>F (- + -)  
g322>a, G108>N (- - -)  
g323>b, G108>N (- - -)  
c327>s  
c329>b, S110>Y (+ + -)  
a330>b, S110>Y (+ + -)  
c331>l, P111>Y (+ + -)  
c332>b, P111>Y (+ + -)  
t333>c, P111>Y (+ + -)  
c334>u |
|  | IGKJ2*01 | 89.74% |  |  |  |

R and S were significant and insignificant nucleotide substitutions, respectively. The (+ + –) indicated of comparison of two amino acid residues (before and after replacement): hydrophobicity, size and physico-chemical characteristics. Percent of homology with embryonic segments was given only for V and J segments of the scFvs
the presence of competitor (A4) at a concentration between 0.0016 to 0.1 mg/ml. Both His-tagged A4 and CBD-fusion A4 were used. For the rabbit anti-CBD Ab (10.8 ng/ml), which bound pSh or T72 and horseradish peroxidase-labelled anti-rabbit conjugates of mouse Ab (1 ng/ml) were used. In case of CBD-fusion A4 the mouse anti-cMyc Ab (1 : 1000), which also bound pSh or T72 and horseradish peroxidase-labelled anti-mouse conjugates of rabbit Ab (1 : 5000) were used.

Surface plasmon resonance

The kinetics of interaction between A4 and pSh/T72 was performed by the surface plasmon resonance instrument ProteOn XPR36 (BioRad Laboratory, Hercules, CA, USA) using purified proteins. A4 was immobilised using the amino groups on the surface of the biochip. pSh or T72 were in flow through the chip surface as analytes at concentrations of 0.266 mM, 0.532 mM, 1.06 mM, 2.13 mM and 0.199 mM, 0.399 mM, 0.798 mM, 9.97 mM, respectively. BSA was immobilised on the biochip as a negative control. The binding of pSh (T72) to BSA was taken into account at final constant calculations. Both His-tagged A4 and CBD-fusion A4 were used in surface plasmon resonance experiments and gave us the same results.

Results

Selection and DNA sequence analysis of specific phages

The selection of scFvs against pSh (mouse idiootype against BP) was carried out by phage display. The naïve combinatorial library of human scFvs was used [24], the size of which was $1.9 \times 10^{10}$ cfu/ml, indicating that the library satisfied with high sequence diversity. The library was panned three times against immobilised pSh to microtiter plate. As a result of analysis of 48 individual bacterial clones, the 14 positive clones with the strongest response to the pSh were selected. The phagemid DNA from the 12 bacterial clones were isolated and sequenced. DNA sequencing of 12 clones showed that all contained inserts phagemid DNA encoding scFvs. Only 7 sequenced DNA samples were unique and the rest of them contained repetitions. Only one clone A4 was chosen for further analysis and work because of the preliminary experiments showed that A4 bound to pSh with high affinity (data not shown).

Analysis of the amino acid and DNA sequences of A4

The analysis of the nucleotide sequences encoding V-, D-, and J-segments of heavy and light chains of A4 was done by the IMGT V-QUEST (Table 1). V-, J-, and D-segments of A4 heavy chain belonged to IGHV1-69*13, IGHJ6*02, and IGHD2-15 family segments, respectively. Light chain of A4 applied to the family of κ-chains. V- and J-segments of A4 light chain belonged to IGKV3-20*01 and IGKJ2*01 family segments, respectively. By comparing the A4 nucleotide sequence with germline segments, it could be assumed that V-segments of the A4 were low
conservative to V germline segments. The percentage identity ranged was 93.75% for the heavy chain and 95.04% for the light chain.

Searching the National Centre for Biotechnology Information database (NCBI, USA) using the BLAST algorithm revealed that A4 was unique and did not have any homology among published antibodies. The amino acid sequence was nearly identical to a sequence in antibody against INF-γ (AHM25306.1) – Max score 360, Query cover 100%, Ident 76% (data not shown).

Figure 1 shows the A4 amino acid sequence consisting of 236 amino acids excluding the 18 amino acid residues linker connecting the heavy and light chains. A4 is composed of 129 amino acid residues of the heavy chain and 107 amino acid residues of the light chain. The Figure 1 shows the analysis of the constant and variable amino acid sequences regions of the heavy and light chains. Interestingly, the shortest of the LCDR2 was only three amino acid residues. The HCDR3 was the longest and consisted of 23 amino acid residues.

The CDR3 region is the most variable part of the scFv, in the formation of which Variable-, Diversity-, and Joining-segments attended. To understand and clarify the recognition of the A4 properties, the maturation mechanisms comprising V/D/J recombination and variability at junctional site of those A4 was studied and compared with their predecessor germline sequences. Table 2 presents an analysis of segment boundaries in the formation of A4. Interestingly, the HCDR3 contained no D-segment. The main part of the HCDR3 was encoded by J-segment and only two amino acids by V-segment. In the case of A4 light chain there was one triplet for Y insertion between V- and J-segments.

Expression and purification of A4

The preliminary experiments showed that the anti-idio
type A4 bound to mouse idiotype antibody pSh with high affinity (data not shown), which is why DNA of A4 was expressed in E. coli and proteins purified in preparative amounts by affinity chromatography on a nickel resin as mentioned in the ‘Materials and methods’ section. Also A4 DNA was subcloned into pTT10 vector for A4 expression as a CBD-fusion. In the last case cellulose was used for A4 purification as indicated in [25]. Figure 2 shows an electrophoretogram of CBD-fusion A4 after SDS-PAGE electrophoresis. The purified A4 migrated as major bands of ~51 kDa consistent with its predicted molecular weights of the processed form of CBD-fusion scFv, i.e. without a leader peptide.

The Affinity of A4

Direct ELISA was used for A4 binding estimation to murine idiotype pSh and human idiotype T72 (Fig. 2). Both His-tagged A4 and CBD-fusion A4 were used in ELISA experiments and gave us the same results (data not shown). pSh was used for phage library screening. However also A4 showed a positive reaction with the T72 in the assay used, despite the fact that the active sites of pSh and T72 were different [25, 26]. A4 binding had a dose-dependent manner in both cases and was very similar.

The optical biosensor ProteOn (Bio-Rad, Hercules, California, USA) was used to determine the dissociation constants for purified A4 and pSh (T72) by surface plasmon resonance. A4 was immobilised onto chip GLM using amino groups. Increasing concentrations of pSh or T72 were in the solution: 0.266 mM, 0.532 mM, 1.06 mM, 2.13 mM and 0.199 mM, 0.399 mM, 0.798 mM, 9.97 mM, respectively. BSA was immobilised on the chip as

![Table 2. The A4 CDR3 formation by gene V-, D-, and J-segments](image)

| V-segment | J-segment |
|-----------|-----------|
| A4 HC     | –MELSSLRSEDTAVYYCAR –DRVPAAMOVSIDYYYYYGMMDV– |
| A4 LC     | –LTISRELPEDFAVYYCQQSN –YTFGGGTKLEIK– |

The CDR3 is in bold. Amino acid chains start with a conservative amino acid residue – L/M89 [30]. HC – heavy chain, LC – light chain

![Fig. 2. The result of ELISA binding of pSh (rhombus) and T72 (foursquare) to microtitter plates immobilized A4. The electrophoresis was on 12.5% acrylamide gels under reducing conditions and stained with Coomassie R-250. A4 was expressed as CBD-fusion and purified on cellulose. A4 migrated as ~51 kDa protein corresponding to protein ladder](image)
The simple 1 : 1 interaction model (A + B = AB) was used for the calculation of values of constants. Table 3 shows the final calculated results for the association rate constants – $k_a$, dissociation rate constants – $k_d$, dissociation constants – $K_d$, and association constants – $K_a$ for A4 binding to pSh and T72. $K_a$ values for A4 to pSh and T72 were very close $4.44 \times 10^{-7}$ M and $5.71 \times 10^{-7}$ M, respectively. It was correlated with ELISA result (Fig. 2) where pSh and T72 binding to A4 curves were almost coincided.

The competitive ELISA was assessed to define anti-idiotype A4 mimicked BP epitope and interacted with active centers of idiotype antibodies against BP (pSh and T72) (Fig. 4). An immunological plate was coated with BP-BSA. The T72 and pSh (concentration at half saturation of the maximum BP binding – IC$_{50}$) were in solution in the presence of increasing competitor concentrations of A4. A4 inhibited the binding of BP-BSA with both T72 and pSh in a dose dependent manner. Moreover, A4 inhibition of T72 binding to BP-BSA was stronger than in the case of pSh to BP-BSA.

**Discussion**

Earlier, high affinity murine scFv Ab1 against BP with $K_a = 0.7 \times 10^{-8}$ M [25, 31] was obtained and monoclonal antibodies with $K_a = 0.68 \times 10^{-10}$ M [32], as well as human scFv against BP with $K_a = 2.93 \times 10^{-7}$ M [26]. The high affinity of human anti-idiotype scFv against BP was described for the first time in the current article.

The A4 against pSh (murine idiotypic against BP) was found after screening of the nadv combinatorial library of scFv genes from human leukocyte, which has been de-
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The authors declare no conflict of interest.

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