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CLONING OF VARIABLE FRAGMENTS OF TUMOR IMMUNOGLOBULIN, ASSEMBLING AND EXPRESSING OF HUMAN SCFV PROTEIN IN *E. COLI* FOR ANTI-IDIOTYPE VACCINATION

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Aim: **Idiotype, the unique part of immunoglobulin molecule expressed on the surface of B-cells, represents a specific antigen for vaccination against lymphoma. We have developed a rapid method for immunoglobulin variable fragments cloning, assembling and expression of recombinant idiotype protein in *Escherichia coli*. *Methods:* PCR with specially designed panel of primers was used for direct amplification of variable regions of tumor immunoglobulin. Overlapping extension PCR, restriction and ligation was applied for assembling and cloning of vaccine construction. Idiotype protein was purified by metal-chelate chromatography. *Results:* Methods of idiotype cloning from lymphoma cells and production of recombinant protein were developed and optimized. Several samples of idiotypic proteins originating from B-cell lines and lymphoma patients were produced. *Conclusion:* The proposed method of vaccine production is relatively cheap, not very laborious and requires as long as 6–7 week to perform. The expressed protein was soluble, did not accumulate in inclusion bodies and harvested at sufficient for vaccination quantity and concentration.**
***Key Words*: immunoglobulin, idiotype, vaccine, lymphoma.**

Development of anti-cancer vaccination has still been limited by the low number of known tumor-associated antigens that might be used as targets for directional immunization. Cells of lymphomas express antigen-specific receptors, surface immunoglobulin for B cell lymphomas and T cell receptor for lymphomas of the T lineage. As tumor has a clonal origin, all cells of lymphoma carry identical immunoglobulin or TCR receptor on their surface. Therefore, variable domains of immunoglobulin (idiotype) can be used as specific antigen for anti-idiotypic vaccination.

The clinical studies of anti-idiotypic vaccination have started since 1992 when Ron Levy and Larry Kwak (Stanford University, USA) first vaccinated follicular lymphoma patients with tumor-derived immunoglobulin [1]. In the last decade numerous clinical trials were performed using hybridoma-prodused immunoglobulin harvested from cell supernatant and showed immunological response and clinical benefit in vaccinated patients [2, 3].

While preparation of individual Ig using rescue hybridoma method is time-consuming and expensive, further studies came to production of recombinant vaccine by cloning variable Ig fragments from DNA of tumor cells [4]. Several methods for expression of Id-protein have been used, including insect cell culture [5], plants [6–8], bacteria *E. coli* [9, 10], or cell-free expression system [11, 12]. These methods require some efforts for purification of protein. Id-protein can be conjugated with immunogenic carrier protein, such as KLH, and administrated to the patient with adjuvant, usually GM-CSF [13]. Alternatively, production of Id-pulsed autologous dendritic cells (DC) can be used for enhancing anti-tumor immune response [14, 2]. Finally, the simplest strategy is nude DNA vaccines [15–17].

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Most methods of recombinant idiotype protein expression and all methods of DNA Id-vaccine utilize cloning of heavy and light immunoglobulin chains variable fragments and their assembling into single chain variable fragment (scFv) [18]. The most difficult problem in that procedure is PCR amplification of the whole variable regions of both immunoglobulin chains due to great number of V_H, V_K and V_L gene segments and their occasional usage in Ig genes rearrangement. To solve this problem, a sophisticated method of anchored-PCR for amplification 5'-end of cDNA was developed [19]. This method implies addition of oligo-dG-tail to the 5'-end of cDNA by enzyme Tdt, first amplification with anchor-primer and subsequent semi-nested amplification with internal primer to the proximal constant region. Anchored-PCR requires optimization to be performed properly. In original publication of F. Osterroth only amplification of IgM heavy chain and IgK light chain was described [19]. Several studies describe amplification of V-regions with specific primers and assembling of scFv [20, 21] for defined immunoglobulin or hybridoma, which is not suitable for broad applications.

In this study, we performed design of specific primers for direct amplification of all possible variable regions of IgM, IgG heavy chains, IgK, IgL light chains without loss or addition of amino acids, and describe method of cloning, assembling scFv and expression of idiotypic protein in bacteria *E. coli*. As well, we realized the ability to add fusion gene of co-stimulator to the construction in the N-part of the protein. Recombinant Id-protein obtained in this way may be used for direct vaccination or loading of DC.

MATERIALS AND METHODS

Cell lines, patient samples, RNA isolation and cDNA synthesis. Four immunoglobulin expressing lymphoma cell lines IM-9, Daudi, RPMI1788 and Na-

malva were used for idiotype cloning. Cells were grown in RPMI-1640 medium with 10% FBS and 2 mM glutamine. 1–5 × 10^6 cells were collected, washed with PBS and lysed for RNA extraction. Tumor cells from patients were obtained from bone marrow, lymph node biopsy or ascitic fluid containing more than 80% of malignant lymphoma cells by histological and/or immunophenotyping analysis. Material from three pediatric patients was used in this study, ascitic fluid from patient 1 with Burkitt's lymphoma, tumor lymph node biopsy from patient 2 with diffuse large B-cell lymphoma and tumor lymph node biopsy from patient 3 with Burkitt's lymphoma. Tissue was disrupted in 2 mL of saline solution with 50 µL of RNAlater (Qiagen) to generate cell suspension. RNA was isolated TRI reagent (Sigma) according to the manufacture's instruction dissolved in sterile water, measured by spectrophotometry and immediately stored at –80°C. 1 µg of total RNA was used for cDNA synthesis with MMLV reverse transcriptase and Oligo-dT.

PCR amplification of Ig variable fragments. PCR amplification was performed for each gene in a semi-nested manner with a specially designed primers panel (details in Results). All primers were designed to unify PCR conditions at 60 °C. PCR reaction was performed in 30 µL with 12.5 pmol of each primer, 200 µM dNTP, 1.5 mM $MgCl_2$ and 1U HF DNA-Polymerase (mix of Pfu and Taq-polymerases, Primetech, Belarus). We used 30 cycles of PCR, but the number of cycles may be reduced to 20 cycles for the first step of amplification to minimize probability of nucleotide replacements due to DNA-polymerase errors. PCR products were examined by 1.5% agarose gel. For the first step of amplification heteroduplex analysis in polyacrylamide gel was applied to discriminate homoduplexes (monoclonal PCR products) from a smear of slowly moving heteroduplexes (derived from polyclonal lymphocytes). This step is useful only for tumor biopsy from patients and not essential for B-cell lines. PCR products were denatured for 5 min at 95 °C and rapidly cooled down to +4 °C to induce duplex formation. Size separation of the generated homoduplexes and heteroduplexes was obtained in 8% non-denaturing polyacrylamide gel.

Molecular cloning and sequencing. Assembled scFv fragment was excised from agarose gel, purified by QIAquick Gel Extraction Kit (Qiagen) and directly ligated into pTZ57R/T vector through T/A overlapping using InsTAclone PCR Cloning Kit (Fermentas). *E. coli* XL1-blue was transformed by calcium chloride method with ligation mix. Selection of blue and white recombinant clones was performed on S-Gal®/LB Agar Blend (Sigma-Aldrich) with ampicillin. Plasmid DNA was recovered from 5–10 clones with EasyPrep Pro Plasmid Miniprep Kit (PerkinElmer). Sequence of insertion was done with plasmid primers pUC19-seq-F GTTTTCCCAGTCACGACGTT and pUC19-seq-R TGTGGAATTGTGAGCGGATA. Sequence reaction was done using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) on genetic analyzer ABI PRISM 3130. Plasmids with correct sequence were digested with NdeI and HindIII, SalI or NotI restrictases (Fermentas) and subcloned into pET24b (Novagen) expression vector. Before final transformation sequence was checked by sequencing with primers pET24b_F TGTGAGCGGATAACAATTCC and pET24b_R TTCCTTTCGGGCTTTGTTAG.

Expression and purification of protein. Recombinant expression vectors with inserted idiotype pET24b-scFv were introduced into bacteria *E. coli* BL21-CodonPlus (DE3)-RIPL and selected by chloramphenicol and kanamycin. Night culture was diluted 1:200 with TB medium, containing 30 mkg/ml kanamycin and cultured at 37 °C until culture reached OD A600 0.6–0.8. Expression was induced by cultivation with 0.5 mM IPTG at 31 °C for 3 hours. Cells were harvested, resuspended in 20 mM Tris-HCl, 0.4 mM PMSF, 0.5 M NaCl (pH 8.0) buffer and disrupted with EmulsiFlex-C5 homogenizer or treated with ultrasound three times for 20 s on ice. Cell debris were separated by 20 min centrifugation 18 500 g and supernatant collected for electrophoresis and protein purification.

Purification of protein was performed by metal-chelate chromatography on columns with Ni-NTA agarose. Binding of protein on the columns, washing and elution was performed in denaturing conditions with 6M urea. Column was washed by 20 mM imidazole solution and protein was eluted by 300 mM imidazole. Urea, salts and imidazole were removed by dialysis [22]. Cell lysate, fraction of column wash and pure protein were analyzed in 12% SDS-PAGE (Laemmli method) and stained with Coomassie Brilliant Blue.

Western blot analysis. Samples of 10 ng of purified protein were run in 12% SDS-PAGE with positive and negative controls. Transfer was done on Semi-dry transport unit (Amersham Biosciences) onto nitrocellulose filter. Filter was pre-stained with reversible dye Ponceau S (Sigma-Aldrich) to check protein bands. Blot was blocked with Western Blocker™ Solution (Sigma-Aldrich), washed with Tween-TBS and treated with primary monoclonal mouse antibodies to 6-His tag (Abcam). Secondary antibodies were rabbit polyclonal to mouse IgG conjugated to HRP (Abcam). Finally blot was stained with diaminobenzene and documented with ChemiDoc™ XRS+ System (Bio-Rad) in visible light. Defined 6His-tagged protein with molecular weight of 17 kDa was used as a positive control. Lines of filter with separated tested and control proteins were also treated with normal mice serum as negative control.

RESULTS

Primers design for PCR-amplification of immunoglobulin variable regions. Most human B-cell lymphoma express immunoglobulin of the IgM isotype, with either kappa or lambda light chain. However, some lymphomas express IgG immunoglobulin [23]. To cover as many lymphomas as possible, we developed extended primer panel for all four immunoglobulin chain types: IgM, IgG, IgK and IgL. Database of all functional V gene segments was collected form http://www.imgt.org website. Immunoglobulin heavy chain V-segments were combined by homology into VH1–6 families and aligned with AlignX service of Vector NTI 9.0 program package

(Invitrogen) and MEGA-4 software. Forward primer was designed to the first 7–8 codons of a VH gene segment avoiding variable and polymorphic nucleotide near the 3'end of the primer. Primer design allowed up to 2 mismatches with germline sequence. An example of primer design for VH1 is shown on Fig. 1.

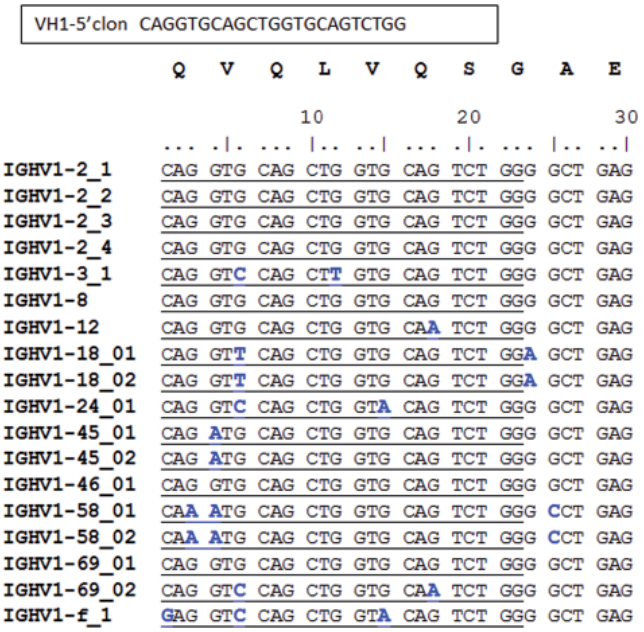

Fig. 1. Primer design for aligned gene segments of VH1 family. Primer sequence is underlined and titled on the top. Variable nucleotides are marked as bold blue letters

The same principle was used for forward primes of all 6 VH families of IgH, all 3 Vk families of IgK and 1–5 VL families of IgL gene. For some IgL V-segments three or four first codons were excluded from PCR amplification to enable optimal primer design. Several VL gene segments belonging to VL6,7,8,9 families were not included due to their very rare occurrence in immunoglobulin molecule.

Two reverse primers were selected for proximal (C1) constant region of IgM, IgG, IgK genes for two steps of semi-nested PCR amplification of variable Ig domains. Distal reverse primer for the first step of PCR is denoted as -3', and proximal primer is denoted as -clon. For IgL gene, one distal IGLC-3' primer was selected for all IGLC regions, but two different proximal primers were selected, one for IGLC1 and another for IGLC2–7 constant regions. Reverse primers to heavy chain include in the cloned fragment a few amino acids (12 a.a. for IgM and 10 a.a. for IgG) on the 5' edge of CH1 segments. This part of constant region was left intentionally to retain with 6His-linker two-domain structure of scFv. The schematic positions of all primers are shown on Fig. 2 and primers sequences are shown in Table 1.

Detection of immunoglobulin expression on tumor cells. Diagnostics of lymphoma includes immunohpenotypic analysis by flow cytometry, including surface immunoglobulin M or G and kappa/lambda light chain detection. Patient's 1 lymphoma cells expressed IgM+/IgK+, patient's 2 — IgG+/IgK+ and patient's 3 — IgM+/IgL+. We also performed immunophenotypic analysis of all B-cell lines kept in our laboratory: REH IgH-/IgK-, Raji IgM+/IgK-, IM-9 IgG+/IgK+, Daudi IgM+/IgK+, RPMI1788 IgM+/IgL+, Namalva IgM+/IgL+. Cell lines REH and Raji were excluded because they do not express mature immunoglobulin on cell surface. Other cell lines: IM-9, Daudi, RPMI1788 and Namalva were used for immunoglobulin V-genes cloning. DNA of all samples used in this study was analyzed by PCR for clonal IgH gene rearrangements as described in our previous publication [24]. This step is not required for vaccine production, but may be useful for rapid identification or verification of V-region and junctional region.

Table 1. Sequences of primers for PCR-amplification of immunoglobulin variable regions

Primer name	Sequence	Position
VH1-5'clon	CAGGTGCAGCTGGTGCAGTCTGG	forward
VH2-5'clon	CAGGTCACCTTGAAGGAGTCTGG	forward
VH3-5'clon	GAGGTGCAGCTGGTGGAGTCTGG	forward
VH4-5'clon	CAGGTGCAGCTGCAGGAGTCGGG	forward
VH5-5'clon	GAGGTGCAGCTGGTGCAGTCTGG	forward
VH6-5'clon	CAGGTACAGCTGCAGCAGTCAGG	forward
Cμ-3'	CTCTCAGGACTGATGGGAAGCC	reverse distal
Cμ-clon	GGAGACGAGGGGGAAAAG	reverse proximal
IgG-3'	GCCTGAGTTCCACGACACC	reverse distal
IgG-clon	CAGGGGGAAGACCGATGG	reverse proximal
Vκ1-5'clon	GACATCCAGATGACCCAGTCTCC	forward
Vκ2/3-5'clon	GATATTGTGATGACCCAGACTCCA	forward
IgκC-3'	CCCCTGTTGAAGCTCTTTGT	reverse distal
IgκC-clon	AGATGGCGGGAAGATGAAG	reverse proximal
VL1_(51)_clon	CAGTCTGTGTTGACGCAGCCGCCCTC	forward
VL1_(36-47)_clon	TCTGTGCTGACTCAGCCACCCTC	forward
VL1_(40)_clon	CAGTCTGTCGTGACGCAGCCGCCCTC	forward
VL2-clon	TCCGTGTCCGGGTCTCCTGGACAGTC	forward
VL3-clon	ACTCAGCCACCCTCGGTGTCAGTG	forward
VL4-clon	TCCTCTGCCTCTGCTTCCCTGGGA	forward
VL5-clon	CAGCCTGTGCTGACTCAGCC	forward
IGLC-3'	GTGTGGCCTTGTTGGCTTG	reverse distal
IGLC2-7_clon	CGAGGGGGCAGCCTTGGG	reverse proximal
IGLC1_clon	AGTGACCGTGGGGTTGGCCTTGGG	reverse proximal

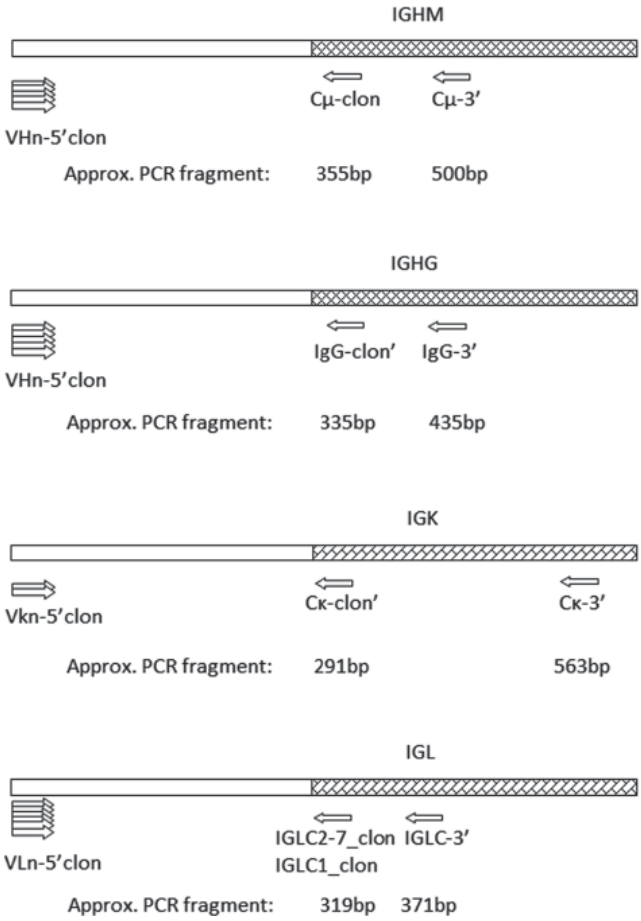

Fig. 2. Scheme of primers positions and approximate length of PCR-products

Amplification of immunoglobulin variable fragments. PCR reactions were performed with primers for identified immunoglobulin chain genes. Separate PCR reactions were done for every VH, Vk or VL primers. Because of similar primer sequence, some PCR reactions showed unspecific amplification, which did not influence cloning (Fig. 3). PCR reactions of the first step which generated clear bands were submitted for the second step with proximal reverse primer. Products were separated in polyacrylamide gel, marked bands of the right size were cut from the gel, DNA eluted and sequenced. Sequence finally proved which V-region corresponds to expressed immunoglobulin. For Daudi cell line, immunoglobulin heavy chain was VH3, and kappa chain was Vk1. Eluted DNA of the right sequence was used as a template for assembling of scFv.

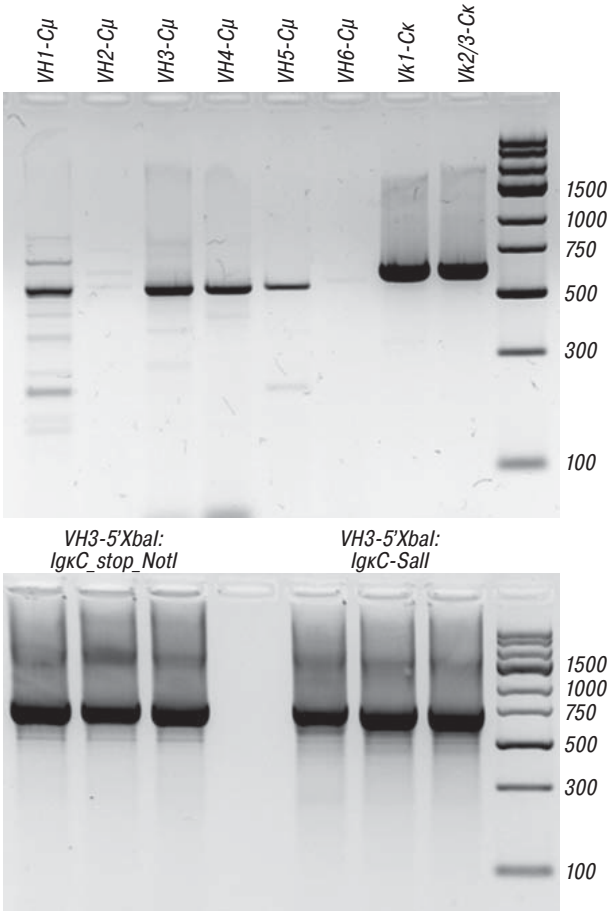

Fig. 3. A. Agarose gel identification of the first step PCR products of IgH and IgK variable regions semi-nested PCR amplification of for Daudi cell line. B. Assembling of scFv by OE-PCR for Daudi cell line in triplicates; primers for cloning are indicated.

Assembling and cloning of scFv. Two variable fragments of heavy and light immunoglobulin chains was further fused together by 6-histidine linker and cloned by two different restriction sites into the expression vector. For that purpose, both fragments were reamplified with primers including restriction sites, start- and stop-codons and 6His-tag. Restriction sites were chosen according to their presence in the MCS of pET24b vector and absence in the consensus regions of used immunoglobulin genes parts. The last principle was not fully applicable due to non-predictable sequence of junctional (CDR3) regions and after hypermutation throughout V-regions. To the beginning of construction the site for NdeI restrictase containing start-codon (CATATG) was added. The end of scFv was designed in two alternative ways. First, the sequence of scFv in open frame finished with SalI or HindIII restriction site. This site may be used to add co-stimulator gene in fusion to scFv as SalI/HindIII — NotI fragment. If no fusion co-stimulator gene is used, transcription will terminate at the pET24b internal stop codon after addition of another 6-His tag to the expressing protein. Second, the sequence of scFv ends with stop-codon and NotI site. Design of several alternative restriction sites was advisedly redundant for a case of casual appearance on of the restriction site sequence inside the segments and to make it possible to clone construction in other vectors. Overall scheme of genetic construction is shown on Fig. 4.

Overlap extension PCR (OE-PCR) was used to fuse variable fragments of heavy and light immunoglobulin chains. For that, 6 histidine codons — CACCATCATCATCACCAC were added to 5'end of V-clon primers of IgK/IgL and reverse complement sequence to the 5'-end of C-clon primers of IgH. We designed different His codons to avoid overlap shift during OE-PCR. The sequences of cloning primers are shown in Table 2.

Each of the purified DNA fragments from the previous step was amplified with appropriate pair of cloning primers. Identical quantity of both PCR products was mixed and subjected to 10 cycles of PCR without primers, applying annealing temperature 55 °C and high-fidelity polymerase. Immediately afterwards another part of PCR mix containing flanking primers and 20 cycles of PCR proceeded with Tan= 65 °C. Fused scFv construct was separated from non-used frag-

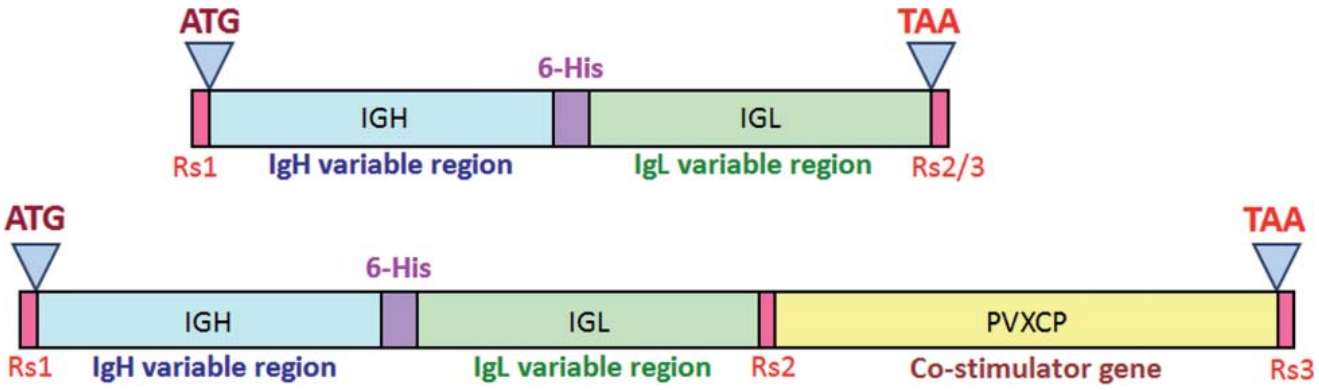

Fig. 4. Scheme of scFv constructions. Restriction site 1 (Rs1) — NdeI, Rs2 — SalI or HindIII, Rs3 — NotI

ments in agarose gel (Fig. 3 *b*), bands of DNA were excised, eluted and directly cloned to pTZ57R/T vector. After proving of the right sequence, scFv was subcloned into pET24b by restriction and ligation.

Table 2. Sequences of primers for scFv assembling and cloning

Forward cloning primers	
VH1-5'-XbaI	GCG TCT AGA CAT ATG CAG GTG CAG CTG GTG CAG TCT GG
VH2-5'-XbaI	GCG TCT AGA CAT ATG CAG GTC ACC TTG AAG GAG TCT GG
VH3-5'-XbaI	GCG TCT AGA CAT ATG GAG GTG CAG CTG GTG GAG TCT GG
VH4-5'-XbaI	GCG TCT AGA CAT ATG CAG GTG CAG CTG CAG GAG TCG GG
VH5-5'-XbaI	GCG TCT AGA CAT ATG GAG GTG CAG CTG GTG CAG TCT GG
VH6-5'-XbaI	GCG TCT AGA CAT ATG CAG GTA CAG CTG CAG CAG TCA GG
Reverse open frame primers	
IgκC-SalI	GCG GTC GAC AGA TGG CGG GAA GAT GAA G
IgκC-HindIII	GCG AAG CTT AGA TGG CGG GAA GAT GAA G
IGLC2-7_SalI	GCG GTC GAC CGA GGG GGC AGC CTT GGG
IGLC2-7_ HindIII	GCG AAG CTT CGA GGG GGC AGC CTT GGG
IGLC1_SalI	CGC GTC GAC AGT GAC CGT GGG GTT GGC CTT GGG
IGLC1_HindIII	GCG AAG CTT AGT GAC CGT GGG GTT GGC CTT GGG
Reverse termination primers	
IGLC2-7_ stop_NotI	GCG GC GGC CGC TTA CGA GGG GGC AGC CTT GGG
IGLC1_stop_ NotI	GCG GC GGC CGC TTA AGT GAC CGT GGG GTT GGC CTT GGG
IgκC_stop_ NotI	GCG GC GGC CGC TTA AGA TGG CGG GAA GAT GAA G
Forward 6-his tag primers	
Vk1-5'tag	CAC CAT CAT CAT CAC CAC GAC ATC CAG ATG ACC CAG TCT CC
Vk2/3-5'tag	CAC CAT CAT CAT CAC CAC GAT ATT GTG ATG ACC CAG ACT CCA
VL1_(51)_tag	CAC CAT CAT CAT CAC CAC CAG TCT GTG TTG ACG CAG CCG
VL1_(36-47)_tag	CAC CAT CAT CAT CAC CAC TCT GTG CTG ACT CAGC CA
VL1_(40)_tag	CAC CAT CAT CAT CAC CAC CAG TCT GTC GTG ACG CAG CCG
VL2-tag	CAC CAT CAT CAT CAC CAC TCC GTG TCC GGG TCT CCT GGA
VL3-tag	CAC CAT CAT CAT CAC CAC ACT CAG CCA CCC TCG GTG TCA GTG
VL4-tag	CAC CAT CAT CAT CAC CAC TCC TCT GCC TCT GCT TCC CTG GGA
VL5-tag	CAC CAT CAT CAT CAC CAC CAG CCT GTG CTG ACT CAG CC
Reverse 6-his tag primers	
Cg-tag	GTG GTG ATG ATG ATG GTG CAG GGG GAA GAC CGA TGG
Cμ-tag	GTG GTG ATG ATG ATG GTG GGA GAC GAG GGG GAA AAG
PVXCP cloning primers	
PVXCP-F-SalI	GCG GTC GAC ATG TCA GCA CCA GCT AGC ACA A
PVXCP-F-HindIII	GCG AAG CTT ATG TCA GCA CCA GCT AGC ACA A
PVXCP-R-NotI	G CGC GGC CGC TTA TGG TGG TGG TAG AGT GAC AAC

For cell lines Daudi, IM-9, RPMI1788 and patients scFv was clones as NdeI — HindIII fragments with additional plasmid 6-His tag at the C-end of protein. Construction of scFv failed for RPMI1788 cell line because of appearance of NdeI restriction site inside IGLV2–23 gene segment. For the last cell line, Namalva, two kinds of construction were made. First ended with the SalI restriction site without stop-codon, which result in addition of 6-His at the 3'-end of scFv, and second terminated close to the NotI site. We also constructed chimeric construction of patient's 1 idiotype and co-stimulating gene of Potato virus X coat protein (PVXCP) [25] (Table 2).

Expression and purification of idiotype protein. Totally, six idiotype proteins were expressed and purified as described in Materials and Methods. Several experiments were employed to optimize induction of protein expression with IPTG. Optimal expression was detected after induction of *E. coli* with 0.4 — 0.5 mM IPTG for 3 hours. SDS-PAGE of cellular protein extracts demonstrated appearance of clear band with an expected molecular weight of 28–30 kDa (Fig. 5).

Our preliminary experiments showed that purification of proteins on Ni-NTA column binding in non-denaturing conditions was confronted with some problems: infirm binding of protein in the column and heavy contamination of eluted fraction with high-molecular weight bacterial proteins. Chromatography of idiotype proteins in denaturing conditions (6M urea) provided firm and specific binding to the Ni-NTA columns and very reliable purification of target protein. All proteins except form scFv went through the column, and after washing, target protein was eluted.

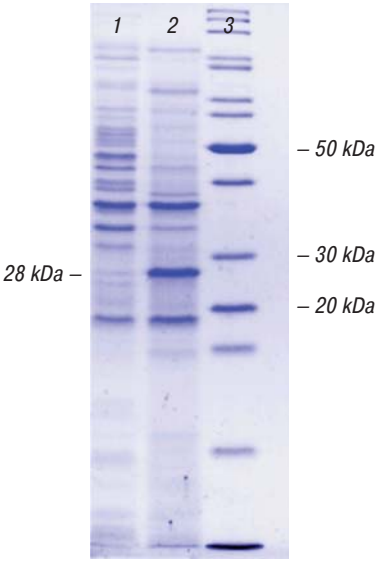

Fig. 5. Induction of Daudi-scFv protein expression in *E. coli* induced by IPTG. 1 — before induction; 2 — after induction; 3 — molecular weight marker

We compared expression of two scFv constructs of Namalva. First, encoding 263 amino acids, was cloned as NdeI-stop-NotI fragment. Second, encoding 278 amino acids, was cloned as NdeI-SalI fragment and included N-tail 6-His tag encoded by the vector. Both proteins were successfully purified (Fig. 6), which means that additional N-terminate 6-His tag did not influence purification efficacy and can be removed. Idiotype proteins were also identified by Western blot analysis using antibodies to 6His-tag. Testing of idiotype protein for IM-9 cell line showed stained band of the right size 28 kDa (Fig. 7).

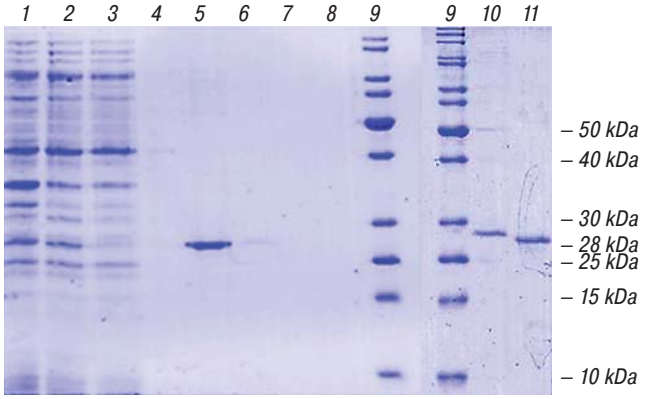

Fig. 6. Fractions of Namalva scFv protein purification. 1 — cell after induction; 2 — supernatant of cell lysate; 3 — lysate passed through the column; 4 — wash of Ni-NTA columns with 20 mM imidazole; 5–8 — fractions of protein elution with 300 mM imidazole; 9 — molecular weight marker; 10 — pure scFv protein (SalI-6-His); 11 — pure scFv protein (stop-NotI)

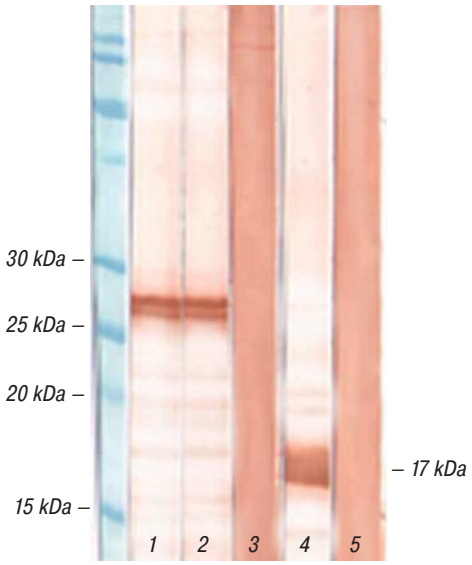

Fig. 7. Western blot of idiotype protein for IM-9 cell line. Lines: 1 — IM-9 protein + anti-6His-AB 1:1000; 2 — IM-9 protein + anti-6His-AB 1:500; 3 — IM-9 protein + mouse serum; 4 — control protein + anti-6His-AB 1:1000; 5 — control protein + mouse serum

DISCUSSION

Series of studies and clinical trials have provided convincing evidence of biological and clinical efficacy as well as of clinical benefit of soluble scFv protein for anti-idiotype vaccination [3, 13, 26, 27]. However, three Phase III randomized clinical trials have failed to achieve their main end points, presumably because of imperfect study design or its conduction [28–30]. At present, customized idiotypic vaccination yet remains a non-approved, experimental approach for patients with lymphoma. Taking into account that hybridoma-derived Id-protein is time consuming and difficult to produce new trials should probably employ 'second-generation vaccine' including recombinant protein, fusion to gene-costimulator and usage of DC for vaccine delivery [4, 28].

The aim of this study was development of a method for the simplest, fast idiotype vaccine production, feasible in basic laboratory of medical centre. Besides the labor and time criteria, method of vaccine production must be applicable to a wide range of patients and different mature B-cell malignances. To realize this aim, two main difficulties have to be overcome: cloning of individual, highly diverse immunoglobulin variable fragments and convenient expression system with protein purification.

Primers were designed for PCR amplification of four immunoglobulin chains: immunoglobulin M and G heavy chains and kappa- and lambda light chains to cover most (>95%) B-cell lymphomas [23]. cDNA was used as a template to allow application of reverse primers to the proximal part of constant region, which possible only after RNA splicing and make it possible to unify primer set. We refused anchored PCR as a complication of the method. Leader sequence of V-segment is not required in bacterial expression system, and does not contain essential antigen determinants for vaccine. Start codon is included in primers for the cloning. Positions of the primers make it possible to amplify the whole sequence of variable regions without loss or addition of amino acids except from 6His-tag in the middle of construction. The use of consensus reverse primer and similarity between forward (V-specific) primers gives some nonspecific amplification, originating from cross-annealing of V-specific primers. But, two steps of PCR, heteroduplex analysis followed by purification of DNA fragment in polyacrylamide gel and sequencing make identification of clonal rearrangements very reliable, even if no data about immunoglobulin expression was obtained by immunophenotyping. Eluted from gel DNA bands was used for amplification with cloning and 6-His tagged primers, accomplishing preparation of both fragments of scFv with 3 rounds of PCR.

Protein produced by hybridoma, transfected mammalian or insect cells takes month or more to prepare. Therefore, choice of the expression system, providing abundant, inexpensive and easily purified vaccine was between bacteria and plants. Attractive method of utilizing tobacco plants for individualized idiotype vaccine production was proposed by A. McCormick and colleagues [6–8, 31]. However, not all clinical laboratories have facilities for plants culturing, and this method require additional 2 weeks for plant infection and accumulation of protein [6].

Expression of protein in bacteria may by the most suitable method, but it also have some serious drawbacks. First, there is some uncertainty if protein of prokaryotic expression is correctly folded and if absence of glycosylation and an inability to form stable disulfide bonds may influence antigen properties of idiotype. Retention of original protein structure depends on the methods of purification. In this study, we made certain that denaturation of protein is necessary for chromatographic purification. In denaturing conditions original bacterial tertiary structure of protein was destroyed and protein was refolded during dialysis. If there is any influence of idiotypic proteins conformation their ability to act as an antigen will be defined after immunological assay of these vaccines. There are some evidences, that scFv proteins expressed in bacteria retain the antigen-binding capacity of original cell-born antibody [32, 21]. However, regardless of tertiary structure of expressed and purified protein, it may be used for loading of DC, as we are going to do in our further experience of vaccine testing and clinical trial. Second problem with expression of protein in bacteria is that protein may accumulate in insoluble form of inclusion bodies. It may be solved by denaturing of protein and its refolding during dialysis. Some studies described that accumulation of allogenic protein in inclusion bodies in bacteria is provoked by overexpression of protein and increased temperature [33, 34]. In our experience that problem was not essential and all proteins were soluble.

In conclusion, we elaborated rapid method of the idiotypic vaccine production by PCR-based cloning and expression of scFv in *E. coli*. Our study was initially intended for DC loading with *E. coli* derived protein, as the most safe and effective way of anti-idiotype immunization.

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