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Basic Investigations

CLONING AND SEQUENCING OF IMMUNOGLOBULIN VARIABLE-REGION GENE OF A MONOCLONAL ANTIBODY SPECIFIC FOR HUMAN HEPATOCARCINOMA

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A murine monoclonal antibody HAb27 specific for human hepatocarcinoma has been developed for radio-immunolocalization in animal models. The isotype of this antibody was IgG1, κ. In the present study, we used a set of oligonucleotide primers to amplify the cDNA of mouse immunoglobulin heavy and light chain variable region genes by the polymerase chain reaction. Sequence analysis of the heavy variable region indicated that the V_H region was highly homologous to the plasmacytoma cell line MOPC21 gene, and closely related to germline genes of the V_HIII family. The J_H region was encoded by the J_H3 gene. For the light chain, the Vκ segment of the antibody showed the highest homology to the germline VκOx1 gene, and the Jκ region was Jκ5.

Key words: Monoclonal antibody, Immunoglobulin variable region gene, PCR, Hepatocarcinoma

Clinical applications of monoclonal antibodies (MAbs) in cancer management include both diagnostic and therapeutic modalities. Their *in vivo* application is limited because in most cases human MAbs of the desired specificity are difficult to prepare. Most available MAbs are derived from mouse hybridomas, and their inherent immunogenicity in patients precludes their long term administration.[1] A new generation of MAbs for human therapy is being produced by recombinant DNA technology. These MAbs are either chimeric[2,3] or fully humanized,[4,5] and their expression in a variety of systems promise to be extremely important. One of these advances has been the development of recombinant single-chain antigen-binding proteins, also termed single-chain Fvs, have recently been constructed and expressed in Escherichia coli and, using a variety of linkers, have been reported to have Ka values approximately one third to one seventh that of the Fab fragment.[6,7] A reduction in the size of the immunoglobulin molecule has been proposed as a mean of increasing tumor penetration, rapid clearance from the blood pool, and decreasing the human anti-murine antibody (HAMA) response.

Murine HAb27 possesses high specificity for human hepatocarcinoma in established cell lines and biopsy tissues.[8] It is minimally reactive with normal tissues, making it a logical candidate for clinical use. Radiolabelled HAb27-IgG has been successfully to image human hepatocarcinomas in nude mice. Here we report the molecular cloning and expression of the variable region gene for the HAb27 antibody, and the sequence information along with the sequence analysis

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is described.

MATERIALS AND METHODS

Hybridoma Cell Line

The hybridoma cell line HAb27 used in this study produced an IgG1-kappa antibody. Details regarding the generation and characterization of this hybridoma cell line has been described.[8] Cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum and 4mM L-glutamine. Cells were maintained at 37 ℃ in a humidified atmosphere with 5% CO_2.

Preparation of RNA And Complementary DNA

Cultured hybridoma cells (up to 10^6/experiment) were washed twice in sterile phosphate-buffered saline (PBS) and suspended in 500 µl of guanidinium isothiocyanate solution. Total cytoplasmic RNA was purified by two extraction with chloroform/isoamyl (49:1 v/v), precipitating at -20 ℃ by the addition of 1/10 vol. of 3M sodium acetate pH 4.8 and 2.5 vols. of ethanol and then collected by centrifugation for 20 minutes at 4 ℃, washed in 70% v/v ethanol, and resuspended in 50 µl of diethyl pyrocarbonate (DEPC) -treated distilled water before storage at -20 ℃. First strand complementary DNA (cDNA) was synthesized at 42 ℃ for 1 hr in a 20 µl reaction volume with oligo-$(d_T)_{15}$ priming. This reaction mixture contained: 8 µl of total RNA, 2 µl of oligo-$(d_T)_{15}$ primer, 4 µl of dNTP mix, 2 µl of 10x first strand buffer, 1 µl RNasin, 1µl of reverse transcriptase (Boerhinger Mannheim, Germany), 2 µl DEPC-treated distilled water.

PCR Amplifications

For amplification with a thermostable DNA polymerase, a 50 µl of reaction mixture containing 5 µl of 10x reaction buffer, 4 µl of dNTP mix and 40 pmol of each primer (V_HBACK 5'GAATTCATGCA-GGTGCAGCTGTTGGAGTCTGG3'; V_HFOR5'GT-CGACTATGAGGAGACGGTGACCAGGGTGCC3'; VκFOR5'GTCGACTAACGTTTGATCTCCAGCTT-GGTC3'). 0.5 µl (2U) Taq DNA polymerase (Boer-hinger Mannheim, Germany), 2 µl of the cDNA and 35 µl distilled water. Following the addition of 50 µl mineral oil, reaction were subjected to 35 temperature cycles comprising 94 ℃ for 50 seconds, 55 ℃ for 60 seconds and 72 ℃ for 60 seconds, with an additional extension step of 10 minutes after the last cycle. Amplification products were assessed by electrophoretic separation of 8 µl aliquots in 1.5% agarose gels and visualization of ethidium bromide stained bands under ultraviolet illumination. The remaining 42 µl of PCR reaction products were extracted with phenol/chloroform. Followed by ethanol precipitation. The samples were taken up in 20 µl of distilled water and stored at -20 ℃.

Nucleotide Sequencing of PCR-amplified V_H and Vκ Gene

The PCR products were digested with EcoRI and SalI and ligated into the sequencing vector M13mp18/mp19. The ligation reaction was used to transfect the competent E. coli JM109 cells, using the calcium chloride method and recombinant M13 plaques were used to prepare ssDNA for sequencing.[9] Dideoxynucleotide chain termination sequencing was carried out using the SEQUENASE T_7 DNA poly-merase according to the manufacturer's protocol (United States Biochemical Corp.). The sequences were analysed by the computer program and compared with GenBank data.

RESULTS

PCR Amplifications

Figure 1 shows the V_H RT-PCR products obtained from the hybridoma cell line (approx. 430 bp). For the V_κ gene of HAb27, the amplified DNA was seen as a major band of the expected size (330 bp, Figure 2). The products from the reactions were analyzed by 1.5% agarose gel electrophoresis, which showed that only one detectable cDNA species was present in each reaction. Moreover, the cDNA fragments produced in these reactions were of the appropriate size for the respective Ig V_H and Vκ.

Nucleotide And Amino Acid Sequences of Immunoglobulin Variable Regions

The PCR products from each reaction were digested with EcoRI and SalI, ligated into M13mp18/

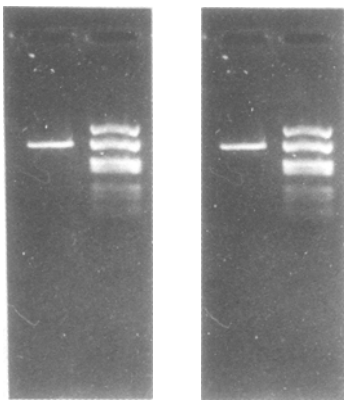

Fig. 1. Amplified cDNA from heavy chain variable region of hybridoma HAb27. The sample (5 µl) was checked on 1.5% agarose gels and stained with ethidium bromide. Lane A: Amplified heavy chain DNA. Lane B: pGEM7zf (+) /HaeIII fragment as marker.

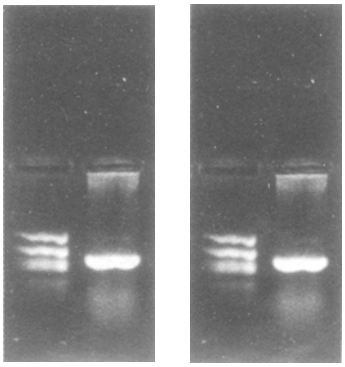

Fig. 2. Amplification of the light chain gene of the mouse hybridoma HAb27. The PCR product was run on a 1.5% agarose gel. Marker sizes are given in bp.

mp19 and sequenced. Figure 3a and 3b show that nucleotide and amino acid sequences of the immunoglobulin heavy and light chain varible regions of HAb27. The results of this analysis confirmed that we had selectively amplified, cloned the heavy and light chain V-regions of an Ig gene, since the sequences obtained a high degree of homology to previous published Ig V-regions in GenBank. Identification of the HAb27 heavy chain V-region sequence as a γ chain was confirmed by the characteristic invariant Cys residues at 23 aa and 97 aa. The V_H segment utilizes the V_HIII family genes. Comparison the V_H segment of HAb27 with the known germline genes in data bank indicated that the V_H gene of HAb27 had the highest homology to mouse plasmacytoma MOPC-21 gene.[10] The J_H segment was encoded by J_H3.

V_H (a)

AGTGAATTCATGCAGGTGCAGCTGTTGGAGTCTGGGGGAGGCTTGGTCAAG

S E F M Q V Q L L E S G G G L V K

CCTGGGGGGTCCCTGAGACTCTCCTGTGCAGCCTCTGGATTCACCTTCAGT

P G G S L R L S C A A S G F T F S

AGCTATAGCATGAACTGGGTCCGCCAGGCTCCAGGGAAGGGGCTGGAGTGG

S Y S M N W V R Q A P G K G L E W

GTCTCATCCATTAGTAGTAGTAGTTACATATACTACGCAGACTCAGTG

V S S I S S S S S Y I Y Y A D S V

AAGGGCCGATTCACCATCTCCAGAGACAACGCCAAGAACTCACTGTACCTG

K G R F T I S R D N A K N S L Y L

CAAATGAACAGCCTGAGAGCCGAGGACACGGCTGTGTATTACTGTGCGAGA

Q M N S L R A E D T A V Y Y C A R

GATGAGGGTTATAGGAGGTATAGCAGCAGCTGGTACGAGCGCAAAAATGAT

D E G Y R R Y S S S W Y E R K N D

AACTACTACTACGGTATGGACGTCTGGGGCCAAGGCACCCTGGTCACC

N Y Y Y Y G M D V W G Q G T L V T

GTCTCCTCATAGTCGACT

V S S • S T

V_K (b)

GAATTCGACATTGTGATGACCCAGTCTCCAGCAATCATGTCTCCATCTCCA

E F D I V M T Q S P A I M S P S P

GGGGAGAAGGTCACCATGACCTGCAGTGCCAGTTCAAGTGTAAGATACATG

G E K V T M T C S A S S S V R Y M

CACTGGTACGAGGAGAAGCCAGGCACCTCTCCCAAAAGATGGATTTTTGAC

H W Y E E K P G Y S P K R W I F D

ACATCCAAACAGGCTTCTGGAGTCCCTGCTCGTTTTAGTGGCAGTGGGTCT

T S K Q A S G V P A R F S G S G S

GGGACCTCTTACTCTCTCACAATCAAGAGCATGGAGGCTGAAGATGCTGCC

G T S Y S L T I K S M E A E D A A

ACTTACTACTGCCTGCAATGGGGTGACCCGTTCACGTTCGGTGCTGGGACC

T Y Y C L Q W G D P F T F G A G T

AAGCTGGAGATCAAACGTTAGTCGACT

K L E I K R • S T

Fig. 3. (a) Nucleotide sequence and the deduce amino acid sequence of HAb27 antibody V_H and (b) V_K. The complementarity determining regions (CDRs) are underlined.

Analysis of complete light chain Variable region nucleotide sequence showed characteristic κ chain invariant Cys residues at 23 aa and 87 aa. Searching of the nucleotide and infered amino acid sequence of

V_κ fragment in EMBL database revealed that it was maximum homology with κ light chain from murine germling V_κ Ox1 gene.[11] The Jκ segment of HAb27 was encoded by Jκ5.

DISCUSSION

The sequencing and cloning of immunoglobulin variable region genes is the first and often rate-limiting step in making genetic engineering antibodies. We here devised a simple and rapid way of cloning these genes via the PCR. By making a systematic comparison of identified nucleotide sequences at the 5' ends of both V_H and V_κ genes that are relatively conserved, we are able to disign primers for PCR amplification based on these sequences and to include restriction sites for forced cloning. In general, the clones could be screened directly by sequencing, with almost all of the recombinants carrying the correct inserts. From the mouse hybridoma, we succeeded in preparing RNA and amplified cDNA that can be sequenced. Errors in the amplified V_H and V_κ genes were readily identified by sequencing clones from two indépendent amplifications. We observed no nucleotide discrepancies on comparing three pairs of about 300 nucleotides. Given our choice of priming sites, it is not possible to determine the exact sequence at both ends of the variable genes, as it is dictated by the primers. However, to some extent, we can reconstruct the sequence in these regions. HAb27 was heavy chain J_H3 and light chain Jκ5, assuming no somatic mutation. Analysis of the V-region sequence revealed that the V_H and V_κ genes of Hab27 is related to the MOPC-21 gene and the germline V_κOx1 gene with 80% and 85% identity in the nucleotide sequence, respectively. The major amino acid residue changes are in the CDR3, an important feature of the "affinity advantage" by the somatic mutation process driven by antigen.[12,13]

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