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Full Length Research Paper

Cloning of pCDNA3-IgG4 and pQE-2-IgG4 human hinge region cDNA for intrasplenic and intraperitoneal immunization

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Human immunoglobulin G4 (hIgG4) is increasingly being used for the detection of various infectious diseases and in allergy-related immunoassays, thus, anti-hIgG4 antibody is of interest in the development of diagnostic tests. The present study was conducted to prepare the plasmid construct of pQE-2-IgG4 for peptide expression and pCDNA3-IgG4 for use in intrasplenic immunization in view of monoclonal antibody production. pQE-2 is a prokaryotic expression vector whereas pCDNA3 is a mammalian expression vector. Some methods were used to compare the efficacy of the immunization routes in raising monoclonal antibody against human IgG4 in Balb/c mice. The cDNA coding for the hinge region of hIgG4 was derived from mRNA from a human blood sample using reverse transcription polymerase chain reaction (RT-PCR). The cDNA sequence was verified by sequencing. The cDNA fragment was then cloned into pQE-2 and pCDNA3, producing pQE-2-IgG4 and pCDNA3-IgG4, respectively. pQE-2 was transformed into *M15* cell to produce the peptide of interest.

Key words: Component, human IgG4, hinge region, cDNA, vector, expression.

INTRODUCTION

In recent years, there is an increasing use of human immunoglobulin G4 (hIgG4) in diagnostics, such as its use in the detection of helminthic diseases (Anantaphruti et al., 2005). As a result of this, the present study was conducted in an attempt to produce recombinant hIgG4 peptide for use as antigen in monoclonal antibody production.

The hinge region of hIgG4 was targeted for the peptide production since this region contains peptide sequences specific to hIgG4 (Lu et al., 2007). Thus, the use of this peptide as antigen for anti-hIgG4 monoclonal antibody (MAb) production would be expected to increase the specificity of the antibody. This in turn may increase the specificity of the test that utilizes the monoclonal antibody.

It is known that, injection of plasmid encoding gene sequences results in the *in vivo* gene expression. Protein will be synthesized in the animal body and the endogenous protein will caused the induction of various immune system mechanisms to produce antibodies against it (Velikovsky et al., 2000).

To compare the efficacy of antibody production using different routes of immunization, namely peptide antigen immunization and DNA immunization, the present study was conducted in an attempt to produce recombinant DNA constructs pCDNA3-IgG4 and pQE-2-IgG4. pQE-2-IgG4 will be used for protein expression in M15 prokaryotic expression cells (producing the peptide for peptide antigen immunization), whereas pCDNA3-IgG4 will be employed in DNA immunization conducted in Balb/c mice for antibody production.

MATERIALS AND METHODS

Bacteria strains and medium

Escherichia coli Top10 was used for transformation of Topo-IgG4 and pGEM-T-IgG4 plasmid constructs. The bacterial strain was obtained from Qiagen, Germany. Luria-bertani (LB) containing 100

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Abbreviation: **hIgG4,** Human immunoglobulin G4; **RT-PCR,** reverse transcription polymerase chain reaction.

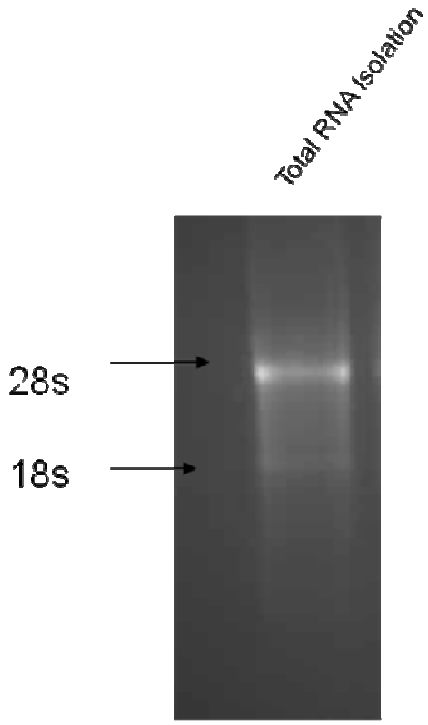

Figure 1. Total RNA isolated from human blood sample was subjected to agarose gel electrophoresis. The bands of 28s and 18s rRNA could be clearly observed (Daruliza et al., unpublished data).

µg/ml ampicillin was used as the culture medium.

Plasmid vectors

Plasmid vectors TOPO and pCDNA3 were purchased from Invitrogen. pGEM-T vector was purchased from Promega and pQE-2 and was purchased from Qiagen, Germany.

Isolation of total RNA

The total RNA was isolated from fresh human blood using Tri-reagent (Fermentas, USA) according to the manufacturer's recommendations. The quantity and quality of RNA were analyzed by denaturing agarose gel electrophoresis. The RNA was stored at -80°C. Informed consent was obtained before the human blood sampling by venipuncture, as required by USM human ethics committee.

Reverse transcription polymerase chain reaction (RT-PCR)

Reverse transcription was performed using a commercial kit (Fermentas, USA). One microgram of the total RNA from the human blood was converted to single stranded cDNA using First Strand cDNA- Synthesis kit. Two microlitre of the resulting product was used as template to amplify hIgG4 hinge region sequence for 35 cycles. One microlitre of total RNA was added into 1 µl of oligo (dT) primer and the total volume was added to 12 µl with the addition of DEPC treated water. The mixture was incubated at 70°C for 5 min. Four microlitres of 5x reaction buffer, 1 µl of Ribolock Ribonuclease inhibitor and 2 µl of 10 mM dNTP were added into the tube. The tube was incubated at 37°C for 5 min followed by the addition of RevertAid M-Mulv Reverse Transcriptase to a final volume of 20 µl. The mixture was then incubated at 42°C for 60 min. The reaction was stopped by heating at 70°C for 10 min and chilled on ice. After the last cycle of amplification, 10 µl of product was stained with ethidium bromide, analyzed using agarose gel electrophoresis and visualized under UV light.

Primers

The hinge region of hIgG4 consists of 20 amino acids. Among them, 14 were identified to be distinctive to hIgG4 (Tan et al., 1990). The human specific amino acid sequence was reported to be SYGPPSAPEFLGGP (Lu et al., 2007). Primers were designed to amplify the region around this hinge region. In addition, the primer design ensured that the amplification product would be flanked by 5'*Nde*I and 3' *Not1* restriction endonuclease cleavage sites to facilitate directional cloning into plasmid pQE-2 and p-CDNA3 vector. The sequences of primers were as follows: Forward primer 3: 5'-catatgagcttgggcacgaagacct-3'; reverse primer 4: 5'-gcggccgccacgcacgtgacctc-3'.

Cloning of PCR products

The human IgG4 cDNA was used as template for PCR cloning. PCR cycle comprised 30 cycles of heating at 94°C for 1 min, annealing at 62.5°C for 1 min, and extension at 72°C for 2 min. The PCR product of approximately 218 bp product was then cloned into TOPO cloning vector (Invitrogen, USA) and pGEM-T cloning vector (Promega, USA) to yield TOPO-IgG4 and pGEM-T-IgG4, respectively. The constructs were subsequently transformed into *Top10 E. coli.* Blue-white colony screening on ampicillin plate was used to select positive clones. Plasmid from positive clones was extracted using plasmid purification kit (Promega, USA).

Recombinant sequence construction

Construction procedure is shown in Figure 1. The TOPO-IgG4 was subjected to digestion with *HindIII* and *XhoI* restriction endo-nucleases, followed by gel purification of the 218 bp desired cassette. The pCDNA3 vector was similarly digested with *HindIII* and *XhoI* restriction endonucleases and gel purified. pGEM-T-IgG4 and pQE-2 were subjected to digestion with *NotI* and *NdeI* restriction endonucleases, followed by gel purification of 218 bp desired cassette. Both insets were ligated to the linearized pCDNA3 and pQE-2 vectors, respectively with T4 ligase in the presence of the desired buffer. The mixture was incubated at 37°C for 1 h. This yielded pCDNA3-IgG4 and pQE-2-IgG4. The resulting construct was then transformed into *TOP10 E. coli* cells and the positive clones were screened by blue white colony screening on ampicillin plate, followed by analysis by *HindIII*, *XhoI, NdeI* and *NotI* restriction endonuclease digestions and confirmed via DNA sequencing.

Sequencing of recombinant constructs

Recombinant plasmids were sequenced using ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (*Perkin Elmer*). For each sample, 5 µl of purified plasmid was mixed with 4 µl of Terminator Ready Reaction Mix (Big Dye) and 1 µl of specific synthesis primer (20 pmol). The cycle sequencing programme was performed for 25 cycles as described by the manufacturer (Perkin Elmer). The sequencing results were analyzed to the known sequences using Bioedit (Version 7) and clustalW.

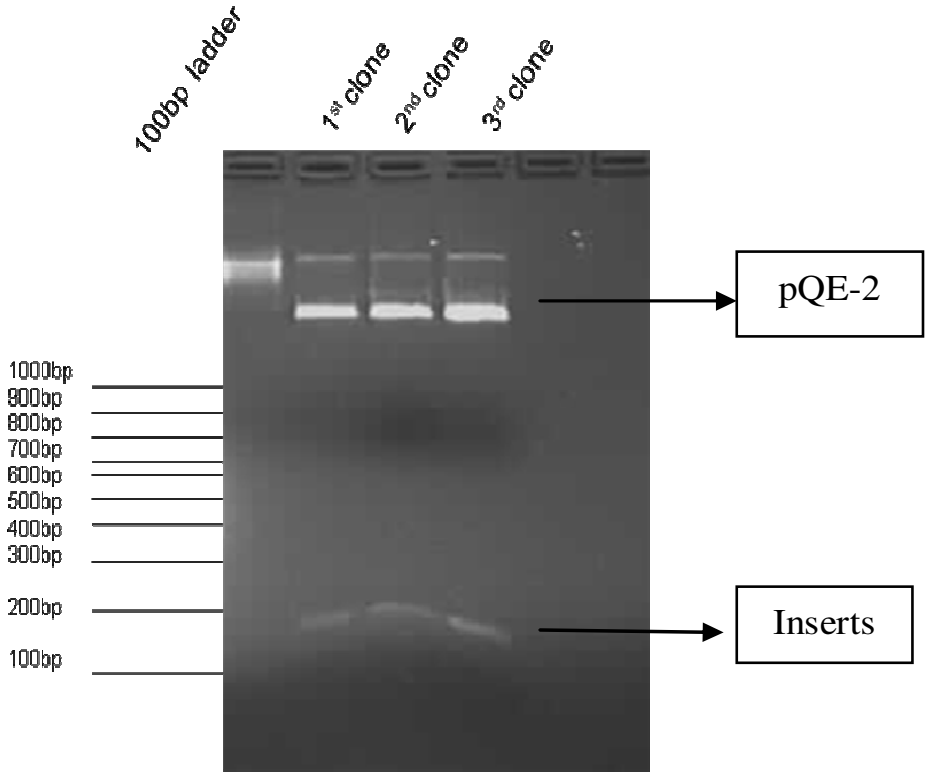

Figure 2. Plasmid DNA isolated from *E. coli* M15 transformed with pQE-2-IgG4 was digested with restriction endonucleases *Not*1 and *Nde*1. Agarose gel electrophoresis result showed the band of pQE-2 (4.8 kb) and the insert (about 204bp) (Daruliza et al., unpublished data).

RESULTS

RNA isolation and RT-PCR

Figure 1 shows the gel electrophoresis result of the total RNA isolated from human blood sample, where clear bands of 28s and 18s rRNA can be observed. The result also shows that there was no obvious degradation of the isolated RNA that was used in the subsequent reverse transcription to generate cDNA. RT-PCR reaction product was analyzed using 1% agarose gel electrophoresis. One cDNA amplicon with an expected size of 218 bp was observed. This was then cloned into Topo and pGEM-T vectors and ultimately yielded TOPO-IgG4 and pGEM-T-IgG4. The sequencing result indicated a sequence with no base mutation, and is as follows:

5'catatgagcttgggcacgaagacctacacctgcaacgtagatcacaagcc
cagcaacaccaaggtggacaagagagttgagtccaaatatggtcccccatg
cccatcatgcccagcacctgagttcctgggggggaccatcagtcttcctgttccc
cccaaaacccaaggacatctcatgatctcccggacccctgaggtcacgtgc
gtggcggccgc 3'

Based on this nucleotide sequence, the corresponding peptide should comprise of 68 residues of the expected amino acid sequence.

Construction of pQE-2-IgG4 expression plasmid DNA

Figure 2 shows the agarose gel electrophoresis result of restriction digestion of pQE-2-IgG4 with *Not*1 and *Nde*1. Two bands representing the vector pQE-2 (4.8 kb) and the insert (218 bp) are shown in each lane loaded with digested DNA extracted from clones 1, 2 and 3, respectively. These pQE-2-IgG4 constructs were sequenced and the results reconfirmed the correct nucleotide sequence of the insert (data not shown).

Construction of pCDNA3-IgG4 expression plasmid DNA

Following the restriction digestion of pCDNA3-IgG4 with *HindIII* and *Xhol*, two bands representing the vector pCDNA3 (5.4 kb) and the insert (218 bp) from a clone were observed on the gel as result from agarose gel electrophoresis (Figure 3). These pCDNA3-IgG4 constructs were sequenced and the results reconfirmed the correct nucleotide sequence of the insert (Figures 4 and 5).

DISCUSSION

The intended recombinant constructs containing the

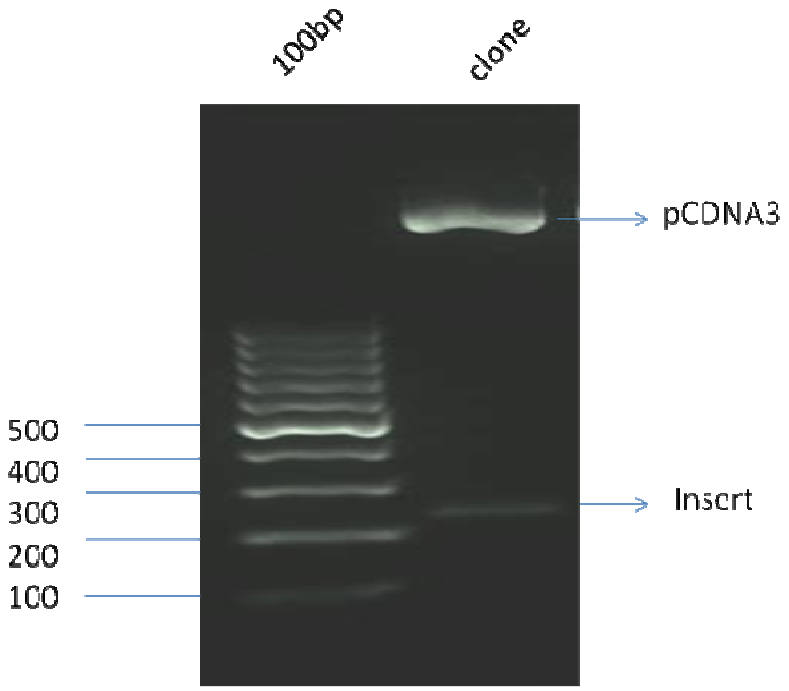

Figure 3. Plasmid DNA isolated from *E. coli Top10* transformed with pCDNA3-IgG4 was digested with restriction endonucleases *HindIII* and *Xhol*. Agarose gel electrophoresis result showed the band of pQE-2 (5.4 kb) and the insert (about 218 bp).

hinge region of hIgG4 were produced. These could be used for subsequent study on the comparison of efficacy of immunization routes used in monoclonal antibody production. Different routes of immunization, namely intrasplenic DNA immunization and intraperitoneal peptide immunization will be carried out and the outcomes would allow the identification of a method of monoclonal antibody production with efficacy. For this, pQE-IgG4 will be transformed into M15 expression cells to express the desired recombinant His-tagged peptide with hinge region sequence specific to hIgG4. With this peptide, the immunization of mice could be performed to produce anti-hIgG4 MAb. However, the removal of histidine tag should first be done to eliminate the possibility of producing non-specific antibody against the His-tag.

The recombinant construct pCDNA3-IgG4 would be used for intrasplenic injection of mice. Assessment of anti-peptide antibody production would be assessed two weeks after injection. If the results were positive, the B-cells from the mice would be taken for antibody-producing hybridoma production.

The capability to produce this hIgG4-specific peptide in unlimited amounts in the laboratory will be very useful in the MAb production, and in other applications. MAb produced against hIgG4 is useful for development of immunoassays to detect helminths infections, in in-house assays and for diagnostic kit production. An example of detection kit that uses anti-hIgG4 is Brugia Rapid^{TM}. The detection of *Brugia malayi* and *Brugia timori* using the kit is based on the principle of binding of the *Bm*R1 recombinant antigen to a filarial-specific hIgG4 antibody in patient sample, followed by binding of the complex to mouse anti-hIgG4 MAb (Rahmah et al., 2001). In the process of the diagnostic kit production, a major cost incurred is the purchase of anti-hIgG4. Since this kit is for detection of brugian filariasis, a neglected tropical disease endemic in poor countries, the ability to produce anti-hIgG4 MAb would enable the kit to be available at a much lower price. Specific IgG4 antibody has also been used as a marker of antigen exposure in allergy, including those caused by food allergy (Tomee et al., 1996; Homburger et al., 1986). The levels of specific IgG4 antibodies have been measured in clinical studies of allergic diseases such as asthma, rhinitis, urticaria and aspergillosis (Till et al., 2004). In addition, allergen-specific IgG4 have also been used in monitoring immunotherapy (Aalberse et al., 1993).

MAbs raised against Fab and Fc regions of the antibody, such as for cancer research, has been previously reported (Li et al., 1998). However, to the best of our knowledge, there is no report thus far on MAb against the antibody hinge region. The peptide of the hinge region is the most specific region for IgG4 molecules, thus, this was cloned for future use to produce anti-hIgG4 MAb. Antibody raised against this region would be expected to be more specific than that raised against the whole antibody.

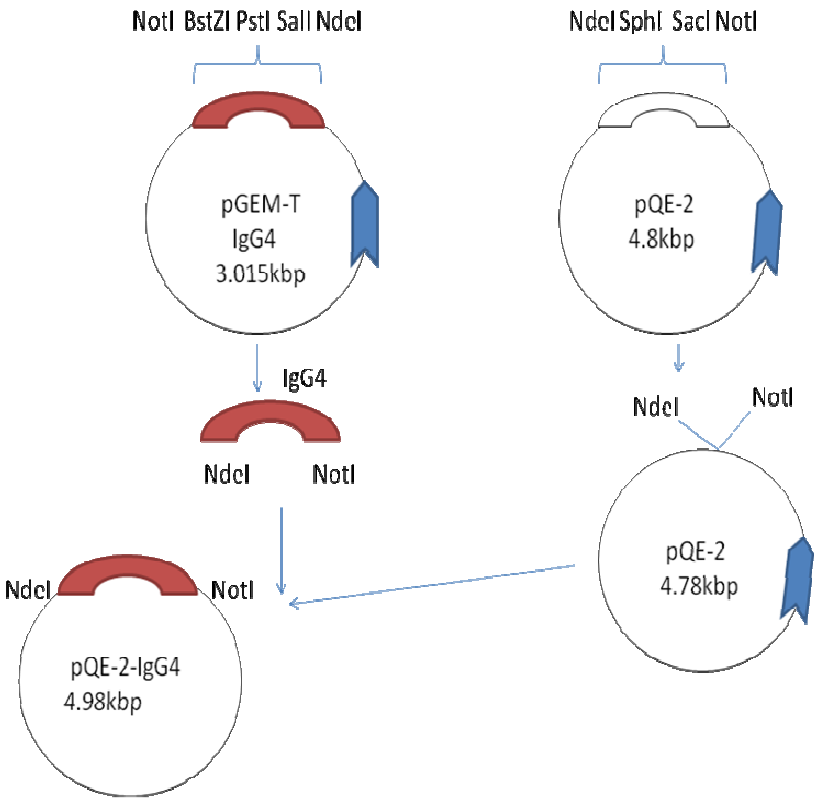

Figure 4. Flow chart for construction of pQE-2-IgG4.

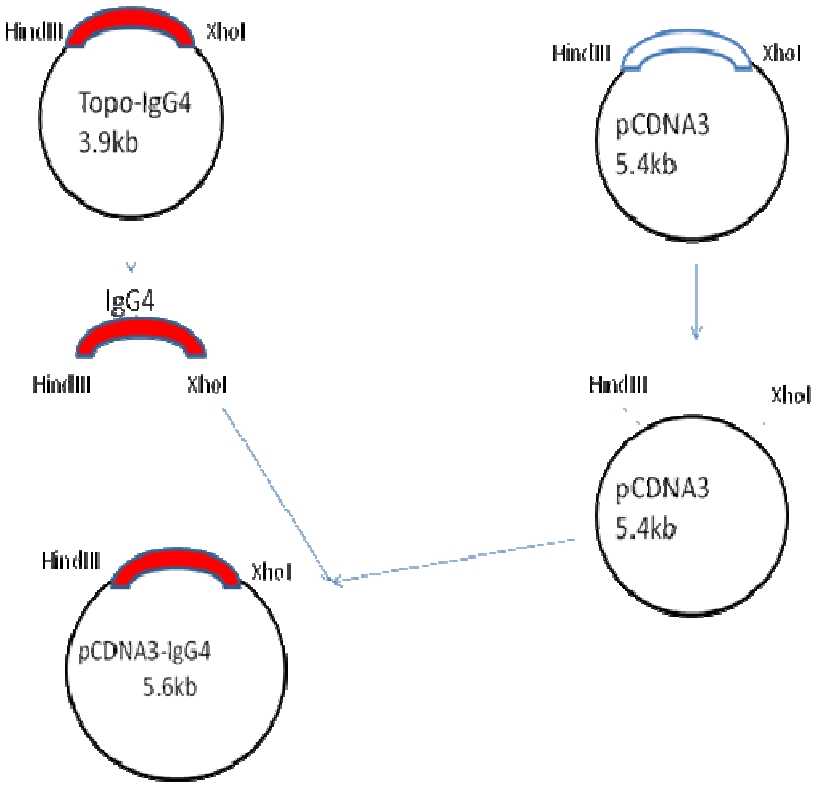

Figure 5. Flow chart for construction of pCDNA3-IgG4.

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