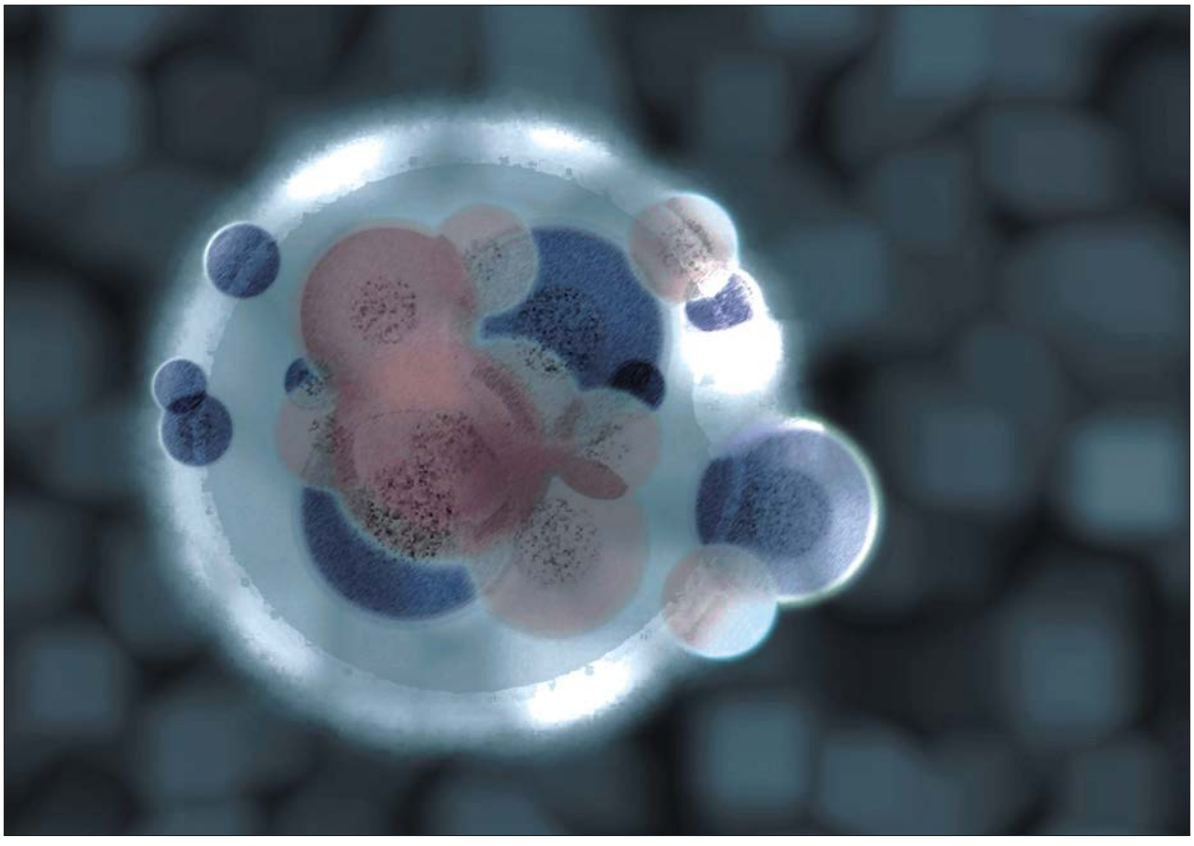

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Production and Purification of Polyclonal Antibody Against Human Kappa Light Chain

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Abstract: The aim of this study was production, purification and HRP conjugation of polyclonal IgG against human kappa light chains. Three 6-month-old New Zealand White rabbits were immunized by human kappa light chain. The IgG fraction was purified by ion-exchange chromatography and labeled with Horse Radish Peroxidase (HRP). Direct ELISA was used to determine the optimum titer of HRP conjugated IgG. The purity of various IgG preparations was about 98%. The optimum dilution of prepared HRP conjugated IgG was 1:16000. The produced antibody has many application in research, clinic and education. This polyclonal antibody can be used for diagnosis and monitoring of free light chain producing diseases.

Key words: Human kappa light chain, rabbit IgG, polyclonal antibody

INTRODUCTION

Polyclonal antibodies are important reagents utilized in a variety of experimental techniques in many fields of biomedical research (de Oliveira and Hirooka, 1999). The ability of polyclonal antibodies to react with multiple epitops of an antigen promote their use in preparation of many immunoassay methods, make them valuable reagents in research and clinical laboratory (Brouwer *et al*., 1993).

Monoclonal free light chains (flc) (traditionally termed Bence Jones proteins) are homogeneous populations of kappa (κ) or lambda (λ) immunoglobulin light chain molecules produced by malignant clones of B-cells. They are important tumor markers for identifying and monitoring patients with Light Chain Multiple Myeloma (LCMM) and light chain amyloid disease (AL) (Bradwell *et al*., 2002). Flc are normally identified in urine, using protein electrophoresis or immunofixation electrophoresis (Keren, 1999).

Serum flc assays are of potential use in diseases of B-cells and plasma cells. Published data is available for many patients with nonsecretory myeloma (NSM) (Drayson *et al*., 2001), LCMM (Abraham *et al*., 2002), MM (Durie, 1986) and Waldenstrom's macroglobulinemia (Bradwell *et al*., 2001). There is also data in AL (Mead *et al*., 2001) and Light Chain Deposition Disease (LCDD) (Bradwell *et al*., 2001). Other diseases such as smoldering myeloma, plasmacytoma, Monoclonal Gammopathy of Undetermined Significance (MGUS), B-cell leukemias and lymphomas are under investigation (Bradwell *et al*., 2002).

This study carried out for production, purification and HRP conjugation of polyclonal IgG against human kappa light chains in rabbits.

MATERIALS AND METHODS

Animals: The study was conducted at Immunology department of Drug Applied Research Center from January 2005 to February 2006. Three 6-month-old New Zealand White rabbits were purchased from Central Animal House of Tabriz University of Medical Sciences for immunization. They were maintained under 12 h light/12 h dark laboratory conditions with free access to the standard food and water. All procedures were performed according to the Guidelines for the Care and Use of Laboratory Animals and approved by the Regional Medical Sciences Research Ethics Committee of Tabriz University of Medical Sciences.

Immunization of rabbits and production of polyclonal antibodies: Three hundred micro litters of prepared human kappa light chain (Sigma) (1 mg mL^{-1}) in PBS (pH 7.4) was emulsified with equal volumes of Freund's complete adjuvant (Sigma) and inoculated intramuscularly (IM) into

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the rabbits. The second and third inoculations were performed on days 21 and 35 with Freund's incomplete adjuvant (Sigma) and the fourth inoculation was done on day 45 without any adjuvant. After the final immunization, blood samples were taken from the rabbits and production of antibody was investigated by double diffusion and ELISA tests.

Purification of rabbit anti-human kappa: Immunized rabbits sera were collected and precipitated by 50% ammonium sulphate. After dialysis against PBS and tris-Phosphate buffer (40 mM tris and 25 mM phosphate, pH 8.2), ion-exchange chromatography was done on a DEAE-Sepharose fast flow (Pharmacia) in a laboratory made column as previously described articles (Majidi *et al*., 2007; Baradaran *et al*., 2006). The eluted proteins were analysed by SDS-PAGE.

SDS-PAGE analysis: The purity of various IgG preparations was checked using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reduced conditions as described by Laemmli (1970). The final concentration of polyacrylamide solution was 13%. Samples were boiled with 2% SDS for 10 min and were loaded on the electrophoresis gel. After separation, the proteins were stained with Coomassie Brilliant Blue G 250 (Wong *et al*., 2000). Destaining was carried out in distilled water.

Conjugation of rabbit IgG with horse radish peroxidase: The conjugation was performed by the periodate method (Nakane and Kawaoi, 1974) with some modifications as previously described studies (Baradaran *et al*., 2006; Majidi *et al*., 2007).

Enzyme Linked Immunosorbent Assay (ELISA): Direct ELISA was designed to determine the titer of HRP conjugated rabbit IgG against human kappa light chain. 10 μg of prepared kappa light chain was added to each well of a 96-well micro titer plate and incubated at 4°C for 24 h. The wells were washed with PBS-Tween (0.05% Tween 20) three times and blocked with blocking solution (PBS-0.5% Tween 20). After a washing step, 100 μL of 1:4000, 1:8000, 1:16000, 1:32000, 1:64000 and 1:12800 dilutions of prepared HRP conjugated anti-human kappa were added to each well. The reaction was developed using 100 μL of 3, 3', 5, 5'- tetramethylbenzidine (TMB) as substrate and the absorbance was determined at 450 nm after stopping the reaction by 5% sulfuric acid (Sigma).

RESULTS

Production of rabbit anti-human kappa: In order to survey production of antibody in rabbits and evaluating effectiveness of immunization, double diffusion and ELISA tests were performed. The relative titer of polyclonal anti- kappa light chain in double diffusion test was 8, which was appeared as a sharp band between antigen and antibody wells. The titer of anti-human kapa determined by ELISA method was 16000.

Purification of rabbit anti-human kappa: Purification of IgG rich fraction from immunized rabbit sera by ammonium sulfate precipitation followed by DEAE ion-exchange chromatography resulted in a highly pure fraction which was appeared as pure bands in SDS-PAGE analysis. The protein content of this fraction was 45 mg which was about one third of primary protein content (Fig. 1).

SDS-PAGE analysis: The results of SDS-PAGE for determining the purity of IgG, which was purified by ion-exchange chromatography has been shown in Fig. 2. A distinct polypeptide band with molecular weight about 50-kDa corresponds to rabbit IgG heavy chains and the diffused bands between molecular weights of 20-30 kDa correspond to rabbit IgG light chain (Fig. 2).

Enzyme linked immunosorbent assay: Direct ELISA was used to determine the optimum titer of conjugated IgG against human kappa produced in rabbits. The optimum dilution of prepared HRP conjugated IgG was found 1:16000.

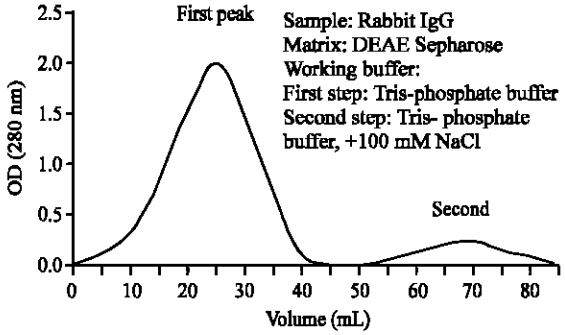

Fig. 1: Chromatographic pattern of purified Rabbit anti-human kappa light chains by ion exchange column with Tris-phosphate buffer, pH 8.1 (first peak) and 100 mM NaCl elution (second peak)

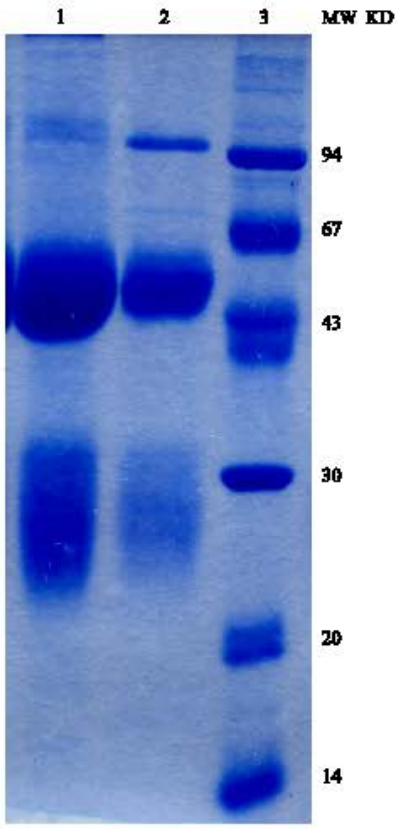

Fig. 2: SDS-PAGE of rabbit anti-human kappa chains, purified by ion exchange chromatography in 13% polyacrylamide gel under reduced conditions, stained with Coomassie Brilliant Blue G-250. First fraction (lane 1), second fraction (lane 2) and low molecular weight markers (lane 3)

DISCUSSION

The potential clinical importance of serum free light chain measurements and improvements in antibody production techniques provoked us to focus on the production of these antibodies.

In this study we used rabbits as host animal for antibody production because is a convenient size, easy to handle and bleed, has a relatively long life span (5-8 years) and produces adequate volumes of high-titer, high-affinity, precipitating antisera (Stills, 1994).

Assays that detect monoclonal immunoglobulin free light chain are important in the diagnosis and monitoring of light-chain-only myeloma, bence Jones myeloma (Bradwell *et al*., 2002; Abraham *et al*., 2002), NSM (Drayson *et al*., 2001) and AL amyloidosis (Abraham *et al*., 2003). They may also be useful in patients producing intact immunoglobulins. Approximately 50% of such patients produce monoclonal light chain in the urine and it was recently shown that >95% of these patients have monoclonal serum FLC at clinical presentation (Mead *et al*., 2004).

Producing polyclonal antibody against human free light chain is important for diagnosis and monitoring of monoclonal free light chain in above mentioned diseases by designing immunoassay techniques such as immunofixation electrophoresis (Thierry *et al*., 1998), immunoelectrophoresis (Ritzmann and Lawrence, 1975) ELISA (Chul *et al*., 1990), western blotting (Walker *et al*., 1987), radioimmunoassay (Robinson *et al*., 1982), turbidimetric (Tillyer *et al*., 1991), latex-enhanced nephelometric (Wakasugi, *et al*., 1995) and Immunohistochemical (Hoshii *et al*., 2006) tests.

After multiple immunizations, blood was collected and ion exchange chromatography was used for antibody purification.

The purification of immunoglobulins presents several practical complications, especially for polyclonal antibody production (Verdoliva *et al*., 2000). We used Ion Exchange Chromatography for purification of rabbit IgG polyclonal antibody. This technique was well established in our laboratory for purification of IgG antibody (Majidi *et al*., 2007; Baradaran *et al*., 2006).

After purification step we obtained a protein with approximate purity of 98%. SDS-PAGE analysis showed that the protein with approximately 50 kDa MW was rabbit IgG heavy chains. The light chain of rabbit IgG was appeared as a diffused band of 20-30 kDa molecular weights. The SDS-PAGE analysis showed that purification of IgG by ion-exchange chromatography resulted in a highly pure product.

Then the purified antibody was conjugated with HRP and an ELISA test designed for determination of conjugated antibody titer. A titer of 16000 indicates the high quality of the product, thus this antibody is highly economical.

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