BIOMEDICAL AND ENVIRONMENTAL SCIENCES **20**, 184-188 (2007)

Production and Characterization of Monoclonal Antibody Against Recombinant Human Erythropoietin[1]

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Objective To produce specific monoclonal antibody (mAb) against recombinant human erythropoietin (rHuEPO) for development of highly efficient methods for erythropoietin detection in biological fluids. **Methods** rHuEPO was covalently coupled with bovine serum albumin (BSA) and the conjugate was used to immunize mice to produce specific mAb against rHuEPO based on hybridoma technology. The obtained F_3-mAb was characterized by enzyme-linked immunosorbent assay (ELISA), SDS-PAGE and Western blot. **Results** The isotype of F_3-mAb was found to be IgM with an affinity constant of 2.1×10^8 L/mol. The competitive ELISA using the obtained IgM showed a broader linear range and lower detection limit compared with previous work. **Conclusions** The modification of rHuEPO was proved to be successful in generating required specific mAb with high avidity to rHuEPO.

Key words: Recombinant human erythropoietin; Monoclonal antibody; IgM; ELISA

INTRODUCTION

Erythropoietin (EPO) is a glycoprotein produced primarily by the kidney[1]. It regulates the proliferation of erythroid progenitor cells and induces their differentiation into mature red blood cells. Declined production of EPO results in anemia[2]. Recombinant human erythropoietin (rHuEPO) has been produced since the human EPO gene was cloned in 1985[3-4] and is used in clinical treatment of anemia. However, rHuEPO is also used as a doping reagent to enhance the oxygen-capacity of blood and thus improve the endurance performance[5-6].

In order to monitor the individual therapy effect in medical treatment and control the abuse of rHuEPO in sports, sensitive determination of the concentration of rHuEPO in biological fluids is of great importance[7-10]. Immunochemical methods have been proved to be powerful tools for such analysis[11]. However, as it was reported and also encountered in our preliminary study, it is difficult to obtain high-affinity antibodies against EPO due to its weak immunogenicity caused by the high degree of conservation in the primary amino acid sequence of EPO between animals[1,12-13]. Injection of a large amount of rHuEPO or direct injection of rHuEPO into the spleen of mice is helpful to enhance the immuno-response, but these approaches often lead to sudden death of the immunized mice because of the side effects of excessive administration of rHuEPO. In our previous study[8], we produced an IgG against rHuEPO using repeated low dose immunization at a longer time interval to stimulate the immuno-response of mice. However, the immunoaffinity constant was only 5.0×10^5 L/mol, which is too low to be used in immunoassay.

In this study, we tried to couple rHuEPO molecules with the carrier protein bovine serum albumin (BSA) to improve the immunogenicity of rHuEPO. By fusion of myeloma cells with splenocytes from mice immunized with the rHuEPO-BSA conjugate, a monoclonal antibody (F_3-mAb) was obtained and characterized by enzyme-linked immunosorbent assay (ELISA), SDS-PAGE and Western blot. The characteristic performance of IgM in immunoassay was also briefly discussed by comparing with IgG.

[1]This work was supported by the National Natural Science Foundation of China (No. 20675006).
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0895-3988/2007
CN 11-2816/Q

MATERIALS AND METHODS

Materials

Recombinant human erythropoietin was produced by Beijing Bio-EPO Biotechnology Corporation Ltd. Each sample vial contains 1.5 mg rHuEPO. Fetal calf serum was purchased from Evergreen Company of Hangzhou (China). Bovine serum albumin (BSA) (fraction V), Freund's adjuvant (complete and incomplete), polyethylene glycol (PEG), horseradish peroxidase (HRP) and mouse monoclonal antibody isotyping reagents were all obtained from Sigma (St. Louis, MO, USA). All other chemicals were of analytical grade.

Instruments

Super clean bench (Beijing Changping Great Wall Equipment & Engineering Company for Air Purification Manager) and CO_2 incubator (Yamato, Model IP-41, Japan) were used in cell culture and fusion. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot were run in Mini-Protein 3 system (Bio-Rad, USA). All data of ELISA were recorded with Tecan Genios Microplate Reader (Austria).

Coupling of rHuEPO With BSA

rHuEPO was coupled with BSA containing glutaraldehyde according to the protocol reported by Baron and Baltimore[14]. Briefly, 1 mL of sodium phosphate buffer (0.2 mol/L, pH 7.5) containing 600 μg BSA was mixed with 1 mL of rHuEPO (1.5 mg/mL). To the mixture, glutaraldehyde (1 mL, 20 mmol/L) was added dropwise. The reaction was kept for an hour at room temperature with occasional stirring and then stopped by adding 2.8 mg of lysine. The conjugate was separated from free BSA and lysine by gel filtration with Sephadex G-75. The rHuEPO-BSA ratio was determined by SDS-PAGE.

Animal Immunizations and Cell Fusion

Female Balb/c mice (6-8 weeks old) were intraperitoneally immunized with the conjugate of rHuEPO-BSA (containing about 50 μg of rHEPO) in complete Freund's adjuvant and boosted twice with the conjugate (containing about 25 μg of rHuEPO) in incomplete Freund's adjuvant at two-week intervals. After the effect of immunization was evaluated by ELISA, the mouse with the highest titer of serum was immunized in spleen with 12.5 μg of rHuEPO. Three days later, the spleen was removed from the spleen-immunized mouse for cell fusion[15]. After collecting and counting, 10^8 spleen cells were diluted with DMEM and mixed with 10^7 myeloma cells (SP2/0) prepared ahead. The mixture was pelleted by centrifugation at 300 rpm for 3 min and suspended in 0.5 mL of HAT medium. Then, 0.8 mL 50% (v/v) warm (37℃) PEG-1500 was added into a 37℃ water bath as the fusing reagent to the cell mixture for 1 min with gentle stirring followed by drawing the cell mixture into the pipet. After holding still for 1.5 min, the mixture was added to a 50-mL tube, 6 mL HAT medium was then added slowly to stop the fusion. Finally the cell mixture was diluted to about 10^6 cells/mL with HAT medium and distributed into each well (0.1 mL/well) of 96-well culture plates to which normal mouse macrophage cells were added ahead as the feeder (0.1 mL/well). Medium in wells was replaced by fresh HAT medium every other day after the cell fusion. Ten days later, HT medium was added to the wells instead of HAT medium. Positive wells were screened by detecting the culture supernatant. Then single clones of positive hybridoma cells were obtained with a limiting dilution technique[16] and expanded stepwise. Some of the positive hybridoma cells were injected into intraperitoneal cavity of BALB/c mice primed in advance with liquid paraffin for antibody production, and the rest were stored at -70℃.

Determination of Isotype and Purification of mAb

Immunoglobulin isotype was determined by ELISA using the mouse monoclonal antibody isotyping kit and the culture supernatants of hybridoma cells.

Ascitic fluid was obtained from Balb/c mice which were previously primed with liquid paraffin before injection of hybridoma cells. After centrifugation, 11.7 mg NaCl and 2.7 mg $CaCl_2$ were added to 1 mL of the supernatants of ascitic fluid. The mixture was filtered using a G-2 glass filter and the filtration was dialyzed against deionized water of 100-fold volume at 4℃ for 15 hours with one change of deionized water. The obtained solution was centrifuged at 12 000 rpm for 30 min, and the supernatant was discarded. The precipitation was resuspended with 1 mL of Tris-HCl (0.1 mol/L, pH 8.0, containing 1 mol/L NaCl). Then the procedures of dialyzation and centrifugation were repeated as described above. Finally, the antibodies were dissolved with 0.5 mL phosphate buffer (0.01 mol/L, pH 7.4, containing 1 mol/L NaCl) and stored at -20℃[17].

Characterization of mAb

The purity and molecular weight of mAb were determined by SDS-PAGE, and 12.5% acrylamide gel was used, the proteins were stained with

Coamassie brilliant blue R-250. The specificity of mAb was confirmed by Western blot. The affinity constant of mAb was measured by non-competitive enzyme immunoassay[18].

Detection of EPO Using Competitive ELISA

Competitive ELISA was performed in 96-well microtiter plates. The wells were washed three times with phosphate buffered saline (PBS) containing 0.05% (v/v) Tween-20 between two steps.

During ELISA, 100 μL of 50 ng/mL rHuEPO was used to coat the wells. After blocking by 300 μL of PBS containing 5% skimmed milk, 50 μL of 10-fold serially diluted rHuEPO and 50 μL of mAb (40 μg/mL) were added to each well and incubated at 37℃ for two hours. Then 100 μL of HRP conjugated goat anti-mouse IgG (L+H) antibody was added. One hour later, the substrate was added and the reaction was terminated with 2 mol/L H_2SO_4 after 15 min of color development.

RESULTS

The SDS-PAGE results of rHuEPO-BSA conjugate are shown in Fig. 1. Four bands were found in the gel with relative molecular weights (Mr) of 210 000, 119 600, 68 000, and 50 000, respectively, indicating that the obtained conjugates were a mixture composed of rHuEPO-BSA and rHuEPO-rHuEPO. The titer of serum was observed to be 1:10000 after 3 immunizations. Of 120 wells, 43 supernatants of hybridoma with cell growth were screened for the production of mAbs against rHuEPO. Among them, 3 wells were found to be positive by ELISA. Finally one stable hybridoma (F_3) was selected.

Isotype analysis indicated that the mAb belonged to IgM subclass. The clone cells were injected into mice to obtain ascitic fluids, from which mAb was purified by dialyzing against deionized water[17]. The relative molecular weight of the mAb was measured by SDS-PAGE (Fig. 2). Light and heavy chains were separated in the gel, and their molecular weights were found to be 25 000 and 72 000, respectively. The immunoreactivity of the F_3-mAb was analyzed by Western blot. The results showed that rHuEPO reacted with mAb and gave a clear band on the membranes from Western blot.

Figure 3 shows the ELISA curves of the measurement of affinity constant. According to the reported method[18], the affinity constant (K_{aff}) could be calculated by the following formula:

$$K_{aff}=\frac{n-1}{2(n[Ab']t-[Ab]t)} \qquad n=\frac{[Ag]t}{[Ag']t}$$

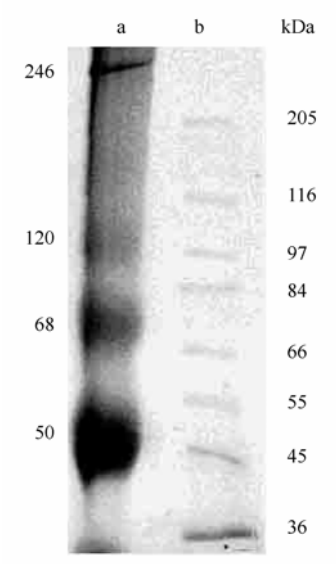

FIG. 1. SDS-PAGE results of the conjugates (rHuEPO-BSA) and high molecular weight standard proteins. Lane a: conjugates; Lane b: high molecular weight markers.

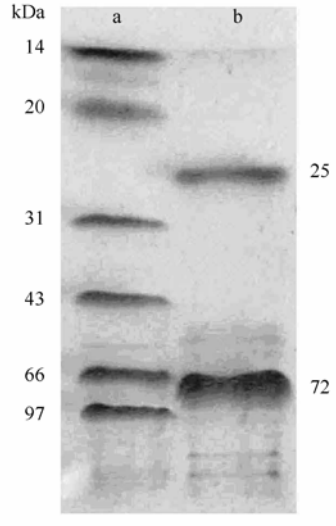

FIG. 2. Separated F_3-mAb and low molecular weight standard proteins in SDS-PAGE. Lane a: low molecular weight markers; Lane b: F_3-mAb.

Where $[Ag]_t$ and $[Ag']_t$ are two different antigen concentrations coated in the wells, while $[Ab']_t$ and $[Ab]_t$ are the measurable total antibody concentrations in the wells at OD-50 (50% of the OD maximum value) and OD-50' for plates coated with $[Ag]_t$ and $[Ag']_t$, respectively. The K_{aff} value of the obtained F_3-mAb was found to be 2.1×10^8 L/mol.

In order to further evaluate the immunoreactivity performance of the obtained IgM to detect rHuEPO, competitive ELISA was performed. For comparison, an anti-rHuEPO mAb B_2 of IgG produced in our laboratory using intact rHuEPO[8] was also applied to the ELISA. All the results are shown in Table 1.

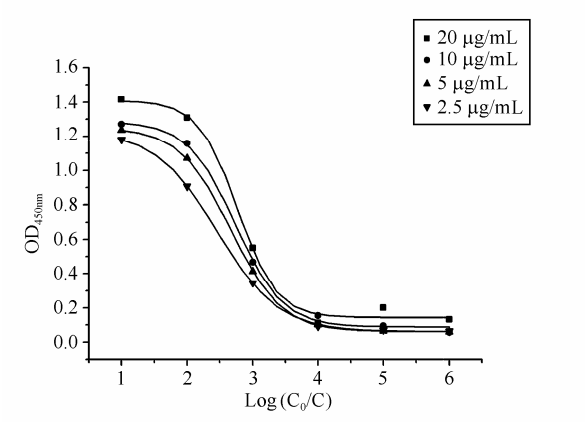

FIG. 3. Dose-response curve of F_3-mAb at different rHuEPO-coating concentrations. The original concentration (C_0) of F_3-mAb was 1 mg/mL.

TABLE 1

Comparison of Obtained IgM With IgG in Competitive ELISA

Isotype	IgM	IgG
K_{aff} (L/mol)	2.1×10^8	5.0×10^5
Regression Equation	A=0.12+0.12 log (C^0_{EPO}/C_{EPO})	A=0.012+0.10 log (C^0_{EPO}/C_{EPO})
Related Coefficient	0.985	0.989
Linear Range	250-50 000 ng/mL	500-15 800 ng/nL
Detection Limit	250 ng/mL	500 ng/mL

DISCUSSION

In view of the degree of homology between the EPO of humans and mice, it is difficult to immunize mice with rHuEPO itself[13]. Antibodies against rHuEPO are commonly produced by immunization of mice with the conjugate of the amino terminus of EPO (residues 1-26) with BSA or other carrier proteins[19-20]. In this study, coupling of intact rHuEPO and BSA was investigated to enhance the immunogenicity of rHuEPO to mice.

Since ε-amino of lysine is the main reactive group in the method of coupling with glutaraldehyde[21], the protein with more lysine residues is generally regarded as the carrier and the protein with less lysine residues would be linked to the carrier as small molecules. Since BSA has 61 lysine residues[22] and rHuEPO has 8 lysine residues[23], one BSA molecule is usually combined with several rHuEPO molecules in the reaction, and the addition of their molecular weights would be equal to the molecule weight of conjugate. In our study, band 1 showed that the ratio of rHuEPO/BSA was 5:1. Band 3 showed that self-coupling of rHuEPO took place in the reaction, for its Mr is equal to 2-fold of the reported value for purified rHuEPO[1]. Since the species with an apparent Mr of 25 000 is found in SDS-PAGE of rHuEPO[24], band 4 might represent the self-coupling products of such species as formed in the procedure of SDS-PAGE due to alteration and degradation of rHuEPO. Band 2 is most probably a BSA molecule combined with two above species represented by bind 4. Based on the above results, the obtained conjugates were a mixture composed of rHuEPO-BSA (5:1 and 2:1) and rHuEPO-rHuEPO.

Since the purity of antigen for immunization of animals is not so rigorous in the production of monoclonal antibodies against a specific antigen in the hybridoma technology, the above conjugates can be used as the antigen to produce mAb against rHuEPO. This was demonstrated by the high titer of serum after 3 immunizations. Since the HRP-conjugated goat anti-mouse IgG antibody belongs to polyclonal antibody against both light and heavy chains of IgG, it is reasonable to react with a mAb with the isotype of IgM.

As a large molecule, IgM is composed of five units linked by bisulphide bonds. In fact, besides the interchain bisulphide bonds, IgM has an additional polypeptide (J chain, Mr 16 000[25]), which is also the joint of units. Generally the two units are first joined together by the J chain, and other units are then combined with these two units by bisulphide bonds. So the J chain should appear in the gel. But in our SDS-PAGE results (Fig. 2), no corresponding band was observed in the gel. It is possible that the J chain was degraded into small peptides in SDS-PAGE. As an intact molecule, the relative molecular weight of F_3-mAb should be the summation of the J chain and five units (each containing two light chains and two heavy chains). So we used 16 000 as the molecular weight of the J chain and the molecular weight of IgM was calculated to be 986 000 Da.

Though in principle an IgM has five units and potential capacity of binding to 10 sites, not all of them are available for combination of the antigen coated on the wells of microtiter plates due to steric hindrance. Therefore the observed affinity constant of IgM is in the normal range of 10^5-10^9 L/mol as reported for most IgG based on the solid-phase ELISA results.

From Table 1 it can be seen that the results of competitive ELISA using IgM had a broader linear range and lower detection limit than those obtained using IgG. The improvement should be due to the higher affinity constant between IgM and rHuEPO, demonstrating the success of the employed coupling strategy to enhance the immunogenicity of rHuEPO to mice.

In summary, modification of rHuEPO by conjugation with BSA is an efficient way to produce high-affinity antibodies against rHuEPO. The

obtained IgM shows a great potentiality for further development of miscellaneous immunochemical analytical methods for rHuEPO in related samples.

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(*Received November* 8, 2005 *Accepted December* 18, 2006)