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Development of polyclonal antibodies for the detection of recombinant human erythropoietin

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Recombinant human erythropoietin (rHuEPO) is detected by using direct pharmacological assays and indirect haematological assays. However, both methods have several limitations including technical challenges and cost-related issues. The aim of this study was to develop polyclonal antibodies against rHuEPO (anti-rHuEPO pAb) that can be used in immunoassays. In this study, we purified anti-rHuEPO pAb that could be used in immunoblotting assays to efficiently detect rHuEPO. Furthermore, these anti-rHuEPO pAb which could also detect rHuEPO that was expressed in a eukaryotic expression system (CHO cells). Thus, the anti-rHuEPO pAb developed in this study may be useful for rHuEPO detection.

Key words: Antibodies, rHuEPO, immunoassays, pAb.

INTRODUCTION

Erythropoietin (EPO) is a glycoprotein hormone that is responsible for the homeostatic regulation of red blood cell production, and consequently increases tissue oxygenation (Lacombe and Mayeux, 1998). Human recombinant erythropoietin (rHuEPO) has been successfully produced in mammalian cells cultures since the late 1980s and has several therapeutic applications such as the treatment of anaemia and polycythemia in patients having chronic kidney disease, acquired immunodeficiency syndrome (AIDS) and cancer (Macdougall and Ashenden, 2009). rHuEPO was used as a performance enhancement drug by athletes participating in endurance sports, has therefore been banned by the World Anti-Doping Agency since 1990 (Reichel and Gmeiner, 2010). Thus, rHuEPO detection methods are used for evaluating blood doping and for disease

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Abbreviations: EPO, Erythropoietin; **rHuEPO,** human recombinant erythropoietin; **CHO,** Chinese hamster ovary; **ELISA,** enzyme linked immuno sorbent assay; **SDS-PAGE,** sodium dodecyl sulfate poly-acrylamide gel electrophoresis.

diagnosis. The increased availability of biosimilars and uncontrolled drugs has resulted in a need for the development of reliable rHuEPO detection methods (Girard et al., 2012).

The rHuEPO detection assays either use a direct pharmacological approach, or an indirect haematological approach. However, both methods have several limitations (Diamanti-Kandarakis et al., 2005) including technical challenges and cost-related issues (Azzazy et al., 2005). Recently, several rHuEPO assays were developed for studying the pathophysiology of anaemia and polycythemia (Lonnberg et al., 2012). Immunoassays that use antibodies against rHuEPO have been immensely useful for studying the structure/function of EPO and for the sensitive detection of EPO in biological fluids (Bornemann et al., 2003; Mi et al., 2005; Sytkowski and Fisher, 1985; Wang et al., 2003).

Cell lines such as the Chinese hamster ovary (CHO) cell line are commonly used for producing recombinant human glycoproteins (Ghaderi et al., 2012). CHO cells have become a popular alternative to animals as an expression system for the efficient production of high quality recombinant proteins. Moreover, the glycosylation machinery of CHO cells is similar to that of human cells (Jeong et al., 2008; Stanley et al., 1996). The objective of this study was to develop polyclonal antibodies for rHuEPO detection.

MATERIALS AND METHODS

Two 6-month-old male New Zealand rabbits were immunized using rHuEPO following a 30-day adaptation period. Five subcutaneous injections were administered in the scapular area of each rabbit, alternating between the right and left sides. The first immunization dose contained 84 µg rHuEPO and complete Freund's adjuvant (Sigma-Aldrich, USA). Subsequent immunizations were performed after 7, 14, 21 and 28 days using rHuEPO (84 µg) and incomplete Freund's adjuvant (Sigma-Aldrich, USA). Prior to immunization, blood was collected to determine the antibody titres. After the last immunization, indirect enzyme linked immuno sorbent assay (ELISA) was used to determine the rHuEPO antibody titres. Hyperimmune sera were obtained from animals with high antibody titre. The hyperimmune serum was stored at -20°C until required for further processing and purification. The antibodies were purified by affinity chromatography using a protein A-Sepharose CL-4B column (GE Healthcare Company, USA) according to the manufacturer's instructions. The animals used in this study were treated in accordance with the guidelines recommended by Colégio Brasileiro de Experimentação Animal. The rHuEPO used in all experiments was EPREX® by Janssen Cilag (Issy-les-Moulinaux, France).

For performing the rHuEPO ELISA, polystyrene ELISA microtitre plates (NuncMaxiSorp®, NalgeNunc International, USA) were coated with rHuEPO (50 ng/well) and incubated overnight at 4°C. Next, the plates were washed with phosphate buffer saline with 0.05% Tween 20 (PBS-T). Then, the wells were treated with blocking buffer (PBS containing 5% skim milk). Serial dilutions of the purified anti-rHuEPO pAb were incubated with rHuEPO-coated wells. To detect the rHuEPO pAb complex, goat anti-rabbit antibody labelled with Ig-peroxidase conjugate (Sigma-Aldrich, USA) was added to the well. A substrate solution containing *o*-phenylenediamine (0.4 mg/mL in 0.1 M citrate buffer, pH 5.0) and 0.03% H_2O_2 was added to the ELISA plate and incubated for 15 min. The reaction was stopped by adding H_2SO_4 (3 N), and the optical densities of the solutions were measured at 492 nm using the VICTOR™ X5 Multilabel Plate Reader (Perkin Elmer, USA).

Immunoblotting was performed to evaluate the specificity and sensitivity of the polyclonal antibodies. Rabbit preimmune sera and anti-EPO rabbit antibody (Sigma-Aldrich, USA) were used as the negative and the positive controls, respectively. rHuEPO solutions with concentrations of 0.05-0.3 µg per well were loaded onto sodium dodecyl sulfate poly-acrylamide gel electrophoresis (SDS-PAGE) gels. For positive and negative controls, rHuEPO concentration of 1 µg per well was used. After electrophoresis, the rHuEPO proteins were transferred onto a nitrocellulose membrane (Millipore, USA) and then incubated at 37°C for 1 h with anti-rHuEPO pAb that was diluted 1:10,000. Next, goat anti-rabbit Ig-peroxidase conjugate (Sigma-Aldrich, USA) was added. The bands were visualized by using substrate/chromogen solution (0.6 mg diaminobenzidine, 0.03% nickel sulfate, 50 mM Tris-HCl at pH 8.0, and 0.03% H_2O_2).

The Chinese hamster ovary (CHO)-K1 cell line was purchased from American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and grown at 37°C in 5% CO_2. Prior to transfection, the cells were seeded in 96-well plates (10^4 cells/well) and incubated until 80% confluence was reached. pTarget/EPO plasmid construct (Collares et al., 2012), pTARGET™ (Promega, USA) and pEGFP (Clontech, USA) were prepared for transfection according to the manufacturer's instructions (Lipofectamine® 2000 Transfection Reagent - Life Technologies,USA). The cells were washed twice with PBS and incubated for 4 h with lipoplex mixture. After 24 h, the lipoplexes were removed by aspiration and the cells were fixed using methanol. The cells were then washed twice with PBS, blocked by using PBS with 10% fetal bovine serum and then incubated with anti-rHuEPO pAb for 2 h. After washing, the cells were incubated with goat anti-rabbit FITC conjugated antibodies (Invitrogen, USA) for 1 h. The fluorescent labels were visualized by using a fluorescence microscope (Olympus BX 71) with an excitation wavelength of 450 nm. CHO cells transfected with pEGFP and stained with a commercially available anti-EPO antibody (Sigma-Aldrich, USA) were used as positive controls; CHO cells transfected with pTARGET and stained with rabbit sera collected before immunization were used as negative controls.

RESULTS AND DISCUSSION

Several manufacturers produce anti-rHuEPO. However, commercially available anti-rHuEPO antibodies remain expensive, and the appearance of new competing manufacturers still has not led to price reduction and solution doping. Considering the high cost for production of monoclonal antibodies, the objective of this study was to produce cost-effective anti-rHuEPO pAb that can be used for immunoblotting and immunofluorescence assays. First, the anti-rHuEPO pAb was purified and used to perform indirect ELISA. The ELISA data indicated that rHuEPO could be efficiently detected using high antibody dilutions (1:10,000). To evaluate the anti-rHuEPO

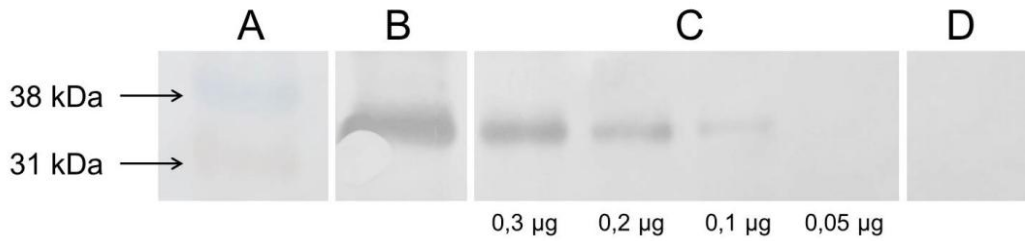

Figure 1. rHuEPO detection by anti-recombinant human erythropoietin polyclonal antibody (rHuEPO pAb) in an immunoblotting assay. **A,** Molecular weight standard; **B,** positive control; **C,** anti-rHuEPO pAb (1:10,000); **D,** negative control.

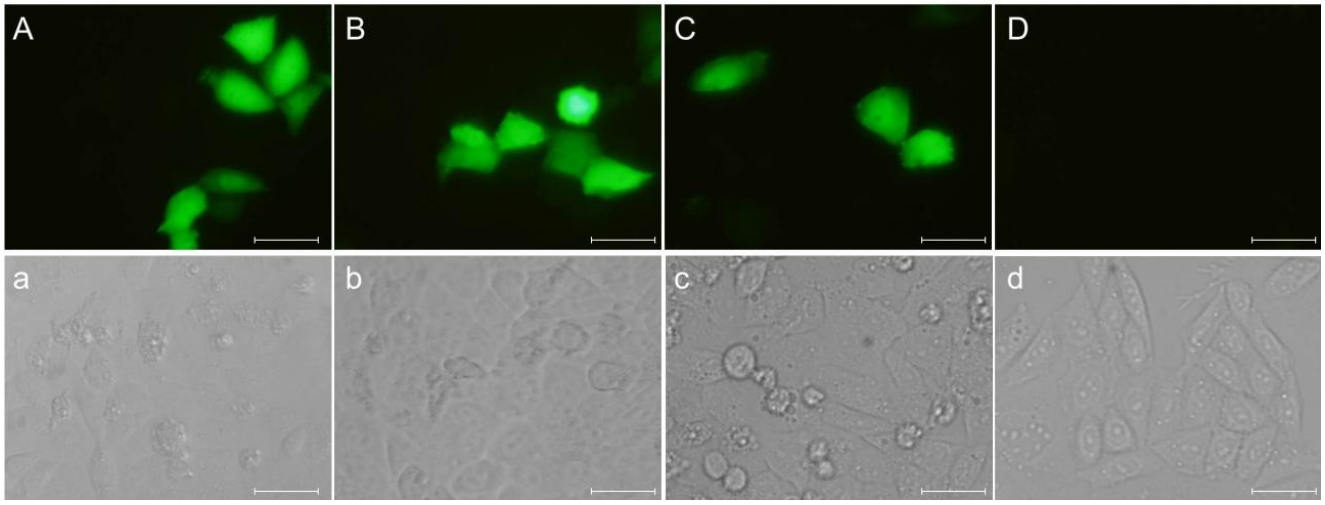

Figure 2. Immunofluorescence analysis of anti-rHuEPO pAb in CHO cells transfected with pTARGET/EPO. Panels: **A,** Transfection control (pEGFP); **B,** specific staining of CHO with commercial anti-EPO antibody; **C,** specific staining of CHO with anti-rHuEPO pAb diluted 1:10,000; **D,** antibody negative control. Phase contrast (a, b, c and d) microphotographs was used. Scale bars represent 100 µm.

pAb detection sensitivity, an immunoblotting assay was performed using various concentrations of rHuEPO. The anti-rHuEPO pAb (1:10,000) detection limit was determined to be 0.1 µg rHuEPO. Bands corresponding to the molecular weight of rHuEPO (30 kDa) were visualized (Figure 1C). Similar results were obtained with the commercial anti-EPO antibody (Figure 1B). In contrast, the rabbit sera obtained before immunization failed to detect rHuEPO (Figure 1D). Since the erythropoietin forms epoetin α and epoetin β are both produced in CHO cell lines and the basis for detecting rHuEPO doping relies on the glycosylation pattern of the protein (Franz, 2009; Girard et al., 2012; Reichel and Gmeiner, 2010), we hypothesized that anti-rHuEPO pAb could detect rHuEPO obtained from a eukaryotic expression system. To evaluate this hypothesis, pTARGET/EPO transfected CHO cells were stained with either anti-rHuEPO pAb (Figure 2C) or the commercial anti-EPO antibody (Figure 2B) to detect rHuEPO expression. The preimmunization sera failed to detect rHuEPO in pTARGET/EPO-transfected CHO cells (Figure 2D) and on pTARGET transfected CHO cells (data not shown).To evaluate the transfection efficiency and rHuEPO expression capability of CHO cells, pEGFP was used as a transfection control (Figure 2A).

The anti-rHuEPO pAb specifically detected rHuEPO by immunofluorescence staining and immunoblotting, which are methods that are commonly used to measure protein expression levels and to evaluate physiological roles of proteins (Ben-Gedalya et al., 2011; Brown et al., 1998; Valdenaire et al., 1999). Polyclonal antibody production offers a rapid and cost-effective alternative to commer-

cially available monoclonal antibodies. In addition, polyclonal antibodies are known to recognize several epitopes on the same antigen and therefore detect antigens more efficiently than monoclonal antibodies. In conclusion, the anti-rHuEPO pAb produced in this study may be useful for various rHuEPO detection assays.

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