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Research Article

Expression and analysis of the glycosylation properties of recombinant human erythropoietin expressed in *Pichia pastoris*

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Abstract

The *Pichia pastoris* expression system was used to produce recombinant human erythropoietin, a protein synthesized by the adult kidney and responsible for the regulation of red blood cell production. The entire recombinant human erythropoietin (*rhEPO*) gene was constructed using the Splicing by Overlap Extension by PCR (SOE-PCR) technique, cloned and expressed through the secretory pathway of the *Pichia* expression system. Recombinant erythropoietin was successfully expressed in *P. pastoris*. The estimated molecular mass of the expressed protein ranged from 32 kDa to 75 kDa, with the variation in size being attributed to the presence of rhEPO glycosylation analogs. A crude functional analysis of the soluble proteins showed that all of the forms were active *in vivo*.

Key words: erythropoietin, glycosylation, *Pichia pastoris*, SOE-PCR.

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Introduction

Erythropoietin (EPO) is a glycoprotein hormone responsible for the regulation of red blood cell production. This hormone triggers the proliferation, differentiation and maturation of bone marrow erythroid precursors into functional erythrocytes when blood oxygen availability is decreased, such as during hypoxia. EPO binds to and activates the receptor on erythroid progenitor cells. The treatment of anemic patients with EPO significantly reduces their dependence on blood transfusions and minimizes potential side effects such as iron overload, infections and adverse reactions to leukocyte antigens. In addition to its role in hematopoeisis, EPO is neuroprotective in the nervous system and can also protect other organs (Genc *et al.*, 2004).

Prior to the 1980s, human EPO for the treatment of kidney failure and other related blood disorders had to be extracted from donors. However, under normal conditions, the expression of EPO is generally low, meaning that many donors are necessary to obtain sufficient material for treatment. Successful cloning of the *epo* gene and subsequent expression in Chinese hamster ovary (CHO) cells led to the production of several types of commercially available recombinant human EPO (rhEPO) for human use. Since then, the possibility of increasing the yield and production efficiency by using other cells, such as tobacco cell lines (Matsumoto *et al.*, 1995), Schneider cell lines (derived from *Drosophila*) (Kim *et al.*, 2005) and mammary gland cells (Zhang *et al.*, 2000), has been explored. However, most of these systems presented serious problems and were not pursued further. Improvement in our understanding of the biological activities of EPO *in vivo* has created a need for technologies that can provide higher yields, prolong the half-life of recombinant EPO and increase the activity of this protein *in vivo*.

In this report, we describe the cloning, expression and analysis of rhEPO produced in the methylotrophic yeast *Pichia pastoris.* Yeasts have long been a model organism for biochemical and genetic studies because of the advantages they offer compared to bacterial systems, including the ease with which they can cultured and maintained, and the fact that they share several important biological characteristics with eukaryotic cells, such as splicing and other processes involved in post-translational modifications. Several yeast species have been used to generate recombinant proteins, including *Saccharomyces cerevisiae*, *Hansenula polymorpha* and *Pichia pastoris* (reviewed in Böer *et al.*, 2007). While *S. cerevisiae* offers similar advantages to other yeasts, *e.g.*, ease of DNA manipulation, shorter cultivation time and the ability to perform post-translational events, the tendency for heterologous proteins to undergo hypermannosylation (with the addition of up to 150

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mannoses) to glycoproteins (Wu *et al.*, 2002) raises the risk of immunogenicity, particularly for therapeutic products. Excessive glycosylation or mannosylation may also hamper efficient secretion of the recombinant protein as the sheer size of the glycogen causes the protein to be retained in the periplasmic membrane, despite the presence of a secretion signal sequence to indicate that the protein should be exported (Kang *et al.*, 1998). For this reason, methylotrophic yeasts such *Pichia pastoris* and *Hansenula polymorpha* are preferred as the overall length of the mannose outer chains is shorter than in *S. cerevisiae* (Kang *et al.*, 1998). Other species, such *Kluyveromyces lactis* and *Yarrowia lipolytica*, are still being investigated for their usefulness as cloning and expression systems.

Materials and Methods

In vitro construction of the *epo* gene

The entire human erythropoietin gene was constructed using the Splicing by Overlap-Extension by PCR (SOE-PCR) technique. Four sets of primers were designed to amplify the four exons of the *epo* gene based on the GenBank nucleotide sequence (GenBank accession number: X02158; Jacob *et al.*, 1985). Primers covering the exon-intron boundaries were designed to contain six nucleotides that were complementary with the adjacent exons in order to facilitate overlapping. An *Eco*RI site (GAATTC) was included in primers at both ends of the target gene to generate sticky ends that would facilitate cloning into the expression vector pPICZαA. The primer sets used are indicated in Table 1.

The four exons of the *epo* gene were initially amplified singly using human genomic DNA as the template. After PCR, the products were purified by gel extraction using a commercial kit (Qiagen, USA). Adjacent exons were assembled in a second PCR reaction by allowing the exons to form partial heteroduplexes in the overlapping regions followed by selective amplification using terminal primers (see Figure 1 for more details). The PCR was done using 1 U of *Pfu* polymerase (Fermentas, Lithuania), 1X of reaction buffer, 1.5 mM Mg^{2+} and 200 μM of each dNTPs (Fermentas, Lithuania). The PCR cycling conditions consisted of 10 cycles of denaturation at 95 °C for 45 s, annealing at 60 °C for 45 s and elongation at 72 °C for 1 min. The selective PCR cycling conditions included an initial denaturation at 95 °C for 3 min, followed by 32 cycles that included denaturation at 95 °C for 45 s, annealing at 60 °C for 45 s and elongation at 72 °C for 1 min, with a final elongation at 72 °C for 5 min. These three steps of PCR were repeated until the entire *epo* gene was obtained.

Cloning of the recombinant *epo* gene

The entire *epo* gene construct was digested with *Eco*RI (Promega, USA) and ligated into the corresponding site in the *Pichia* plasmid expression vector pPICZαA. This vector has a highly-inducible promoter (AOX1) and secretion signal (α-factor). The recombinant plasmids were transformed into *E. coli* (TOP10F' strain) for scale-up isolation. The recombinant pPICZαA was then linearized by treatment with *Sal*I (Fermentas, Lithuania) and transformed chemically into *P. pastoris* (X-33 strain) by following the protocol in the *Pichia* expression manual (Invitrogen, USA). The nucleotide sequence of the recombinant *epo* gene construct was confirmed by DNA sequencing.

Two recombinant *epo* genes (*pPICZα-rhEPO-Stop* and *pPICZα-rhEPO-His*) were constructed (Figure 2). A stop codon (TGA) was introduced into *pPICZα-rhEPO-Stop* to produce a mature EPO of 165 amino acids. To express a fusion protein containing the polyhistidine tag (*pPICZα-rhEPO-His*) the *rhEPO* gene was cloned in frame with the C-terminal peptide. Both vectors contained a native α-factor signal sequence that provides efficient secretion of most proteins from *P. pastoris*.

Expression of the recombinant *epo* gene in *P. pastoris*

For small scale culture, a single colony or frozen stock culture was grown overnight with shaking (220 rpm, 30 °C) in 10 mL of BMGY (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base, 4 x 10^{-5} % biotin and 1% glycerol). The cells were then pelleted by centrifugation (3000 *g*, 10 min, room temperature) and transferred to 50 mL of BMMY (1% yeast ex-

Table 1 - The sequences of primers used in overlapping extension. The underlined sequences indicate the *Eco*RI site.

Exon	Primers	Sequences
Exon 1	EPO-F	5'-GTCGACGCGGCCGCG<u>GAATTC</u> GCC CCA CCA CGC CTC ATC TGT-3'
	$EPO\text{-}1R_o$	5'-GCC CGT CGT GAT ATT CTC GGC CTC CTT G-3'
Exon 2	$EPO\text{-}2F_o$	5'-ATC ACG ACG GGC TGT GCT GAA CAC TGC -3'
	$EPO\text{-}2R_o$	5'-CCC GAC CTC CAT CCT CTT CCA GGC ATA G-3'
Exon 3	$EPO\text{-}3F_o$	5'-ATG GAG GTC GGG CAG CAG GCC GTA GAA G-3'
	$EPO\text{-}3R_o$	5'-TTC CTT CTG GGC TCC CAG AGC CCG AAG C-3'
Exon 4	$EPO\text{-}4F_o$	5'-GCC CAG AAG GAA GCC ATC TCC CCT CCA G-3'
	EPO-Stop	5'-GCGCTACGTTCACAA<u>GAATTC</u> TCA TCT GTC CCC TGT CCT GCA GGC CT-3'
	EPO-His	5'-GCGCTACGTTCACAA<u>GAATTC</u> CA TCT GTC CCC TGT CCT GCA GGC CT-3'

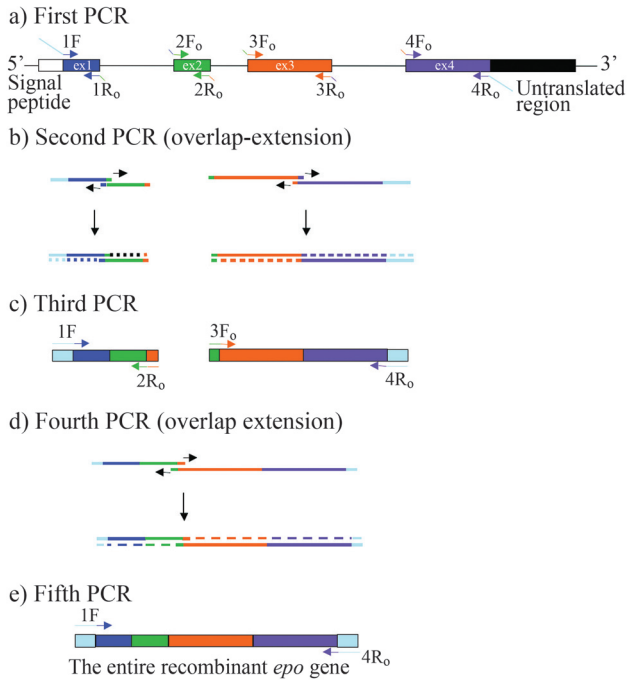

Figure 1 - Construction of the *epo* gene via SOE-PCR.

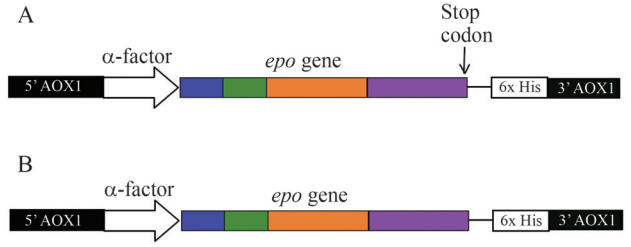

Figure 2 - Constructs for erythropoietin production. A: pPICZα-rhEPO-Stop; B: pPICZα-rhEPO-His (5'AOX1 - alcohol oxidase promoter; α-factor - secretion signal peptide; 6x His - C-terminal polyhistidine tag; 3'AOX1 - transcriptional termination sequence).

tract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base, 4 x 10^{-5}% biotin and 1% methanol) in a 250 mL flask. The culture was incubated at 30 °C with shaking (220 rpm) and at different time intervals the supernatants were collected by centrifugation. The supernatants were precipitated by adding three volumes of acetone and incubated at -20 °C overnight. The mixtures were then centrifuged (10,000 rpm, 4 °C) and the pellets then dissolved in phosphate-buffered saline (PBS).

SDS-PAGE and western blotting

SDS-PAGE was done in 12.5% polyacrylamide gels. After electrophoresis, the gels were either stained with silver or the proteins were transferred to a nitrocellulose membrane (Amersham Biosciences, USA) for western blotting, as described in the standard protocol (Bio-Rad, USA). The recombinant protein was detected using monoclonal anti-human EPO antibody (diluted 1:8000; R&D Systems, USA) and a secondary anti-mouse antibody conjugated with alkaline phosphatase (diluted 1:6000; Sigma, USA). The proteins were viewed with Western Blue® stabilized substrate for alkaline phosphatase (Promega, USA). Commercial recombinant EPO (cEPO) used as standard in electrophoretic analysis was produced in CHO cells (kindly provided by Duopharma Biotech Bhd., Malaysia).

Purification

rhEPO-His was concentrated and purified on a His GraviTrap column containing precharged Ni Sepharose 6 Fast Flow (Amersham Biosciences, USA). After loading, the column was washed with 20 mM imidazole and eluted with 200 mM imidazole in 20 mM sodium phosphate, pH 7.4, containing 500 mM NaCl.

Fractionation

rhEPO-Stop and rhEPO-His were fractionated in a Mini Prep cell (Bio-Rad, USA) with a 10% resolving gel. The apparatus was assembled according to the manufacturer's instructions and electrophoresis was done at 200 V (~3-6 mA) for 2-2.5 h. The eluted fractions (200-250 µL each) were collected every 15 min. All of the fractions were analyzed by SDS-PAGE and western blotting.

Deglycosylation

rhEPO-His and rhEPO-stop were digested with PNGase F (New England Biolabs, UK) and Endo H (New England Biolabs, UK), according to their manufacturer's instructions. Deglycosylated rhEPOs were analyzed by western blotting. cEPO treated with these enzymes was used as a positive control.

Assessment of hematopoietic activity *in vivo*

Female ICR mice (provided by the Animal House of the University of Malaya) were used to test the hematopoietic activity of rhEPO produced in *P. pastoris*. Each mouse was injected subcutaneously with 0.25 mL of sample (acetone-precipitated supernatant of rhEPO cultures) for three consecutive days. Three mice (6-8 weeks old) were used for each treatment and two groups of mice were used as controls (one of these two groups was treated with PBS and the other received no treatment). Blood samples were collected into 5% sodium EDTA on the fourth day after treatment. An equal volume of blood was mixed with new methylene blue and incubated at 37 °C for 1 h. Seven microlitres of this blood-dye mixture was then used to prepare smears on glass slides. Five slides were prepared for each mouse (total of 15 slides per treatment since there were three mice per group). Reticulocytes were counted with the aid of a microscope (at 100X magnification) in five randomly selected areas of each slide and their number expressed as a relative to the total number of red blood cells observed.

Statistical analysis

The results were expressed as the mean ± SEM, where appropriate. Statistical comparisons were done using Students *t*-test, with a value of $p < 0.01$ indicating significance.

Results and Discussion

Expression and characterisation of rhEPO

Four exons (105, 99, 192 and 183 base pairs) of the *epo* gene were successfully ligated via SOE-PCR to produce the full length gene of 543 base pairs (Figure 3), with *Eco*RI sites incorporated at both terminals. SOE-PCR is a simple, cost-effective tool for recombinant gene construction, especially for EPO which generally has a low expression in tissues other than the kidney. This procedure circumvents the laborious work of mRNA extraction from human kidney cells. The fidelity of PCR and subsequent gene construction steps was confirmed by sequence analysis, which revealed that the recombinant constructs had exactly the same sequence as published by Jacob *et al.* (1985). By using this approach, the overlapping region between two adjacent exons could be minimized to 12 bp without any reduction in splicing efficiency. This overlap was shorter than those reported previously by using the overlap-extension method (Wurch *et al.*, 2000; Davidson *et al.*, 2002; Ailenberg *et al.*, 2005).

Both versions of recombinant EPO, *i.e.*, with and without a polyhistidine tag (rhEPO-His and rhEPO-Stop), were successfully expressed, but at very low levels. A series of optimized culture conditions was used to achieve higher yields (data not shown). In addition, by lowering the culture temperature, a mixture of glycoisoforms was produced that resulted in the appearance of a broad smear in subsequent western blots (Figure 4, lanes 3 and 5). The molecular mass of rhEPO-Stop ranged from 32-75 kDa while that of rhEPO-His ranged from 37-75 kDa, all of which were larger than that observed for cEPO (~32-37 kDa). Native human EPO is highly glycosylated

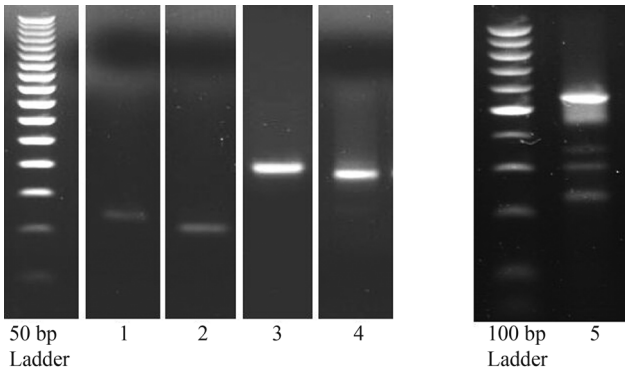

Figure 3 - Construction of the entire *epo* gene via SOE-PCR. Lanes 1-4: Amplification of exons 1, 2, 3 and 4 (105, 99, 192 and 183 bp), respectively; lane 5: Amplification of the entire *epo* gene (543 bp); the smaller non-specific bands were eliminated by gel purification.

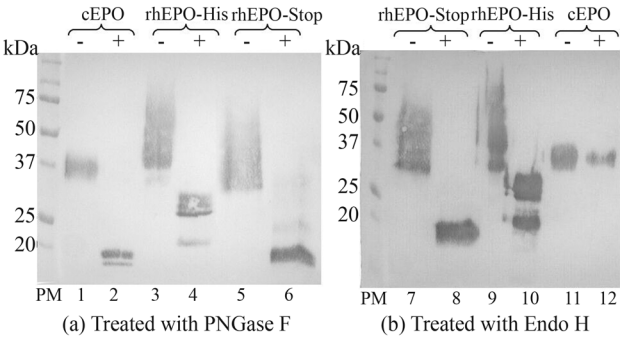

Figure 4 - Western blots of cEPO, rhEPO-His and rhEPO-Stop after treatment with (A) PNGase F and (B) Endo H. Treatment with PNGase F or Endo H is indicated with (+) and no treatment is indicated with (-). PM - protein molecular mass markers.

(~32-37 kDa) and approximately 30% of its molecular mass is attributed to the carbohydrate chains (Dordal *et al.*, 1985). Our results showed that glycosylation in rhEPO expressed in *P. pastoris* accounted for ~30%-70% of the total molecular mass. This phenomenon is not unusual since hyperglycosylation or hypermannosylation is frequent in yeast expression systems (Wu *et al.*, 2002). Various studies have shown that glycosylation in *P. pastoris* generally results in heterologous recombinant proteins that have a greater molecular mass than the corresponding proteins produced in mammalian expression systems (Braren *et al.*, 2007). However, there are cases in which the recombinant proteins produced in *P. pastoris* have a lower molecular mass than those produced in mammalian systems. Sadhukhan and Sen (1996) postulated that this may reflect the absence of other post-translational modification processes in yeast, *e.g.*, phosphorylation, sulphation and sialylation. Examples of this would be the envelope glycoproteins of classic swine fever virus (E^{rns}) which, when expressed in *P. pastoris*, has a lower molecular mass than expected (Huang *et al.*, 2006).

To investigate the variation in glycosylation of the recombinant proteins, the two recombinant EPOs were treated with glycosidase (PNGase F) to release the N-linked carbohydrates. Deglycosylation reduced the molecular mass of both recombinant EPOs. As predicted from the amino acid sequence, the molecular mass of the polypeptide backbone of cEPO and rhEPO-Stop was similar, *i.e.*, ~18 kDa (Figure 4A). For rhEPO-His, the predicted molecular mass of the polypeptide backbone was ~23 kDa because of the additional 44 amino acids in the C-terminal of the protein that included the polyhistidine tag for purification. Figure 4A also shows that two bands were observed for commercial EPO, with the upper band corresponding to the EPO protein with O-linked carbohydrate while the lower band was the non-glycosylated EPO (Elliott *et al.*, 1994). Two bands were also observed for digested rhEPO-His but the molecular masses differed when compared to cEPO. We postulated that the lower band (23 kDa) was the polypeptide backbone of rhEPO-His, while the upper band

(~26-31 kDa) was deglycosylated EPO with O-linked oligosaccharides. Although this suggestion requires confirmation similar results were obtained after prolonged deglycosylation (for at least 24 h), suggesting that the presence of the larger band was not due to incomplete deglycosylation (data not shown).

To further assess the differences in glycosylation between cEPO and rhEPOs the N-linked glycans was also removed using the enzyme Endo H, which only cleaves high mannose sugar chains from N-linked glycoproteins. As expected, digestion of rhEPO-Stop and rhEPO-His with Endo H produced results similar to those obtained with PNGase F, indicating that *Pichia*-expressed rhEPO contained high mannoses only and no complex sugars (Figure 4A,B). However, Endo H digestion appeared to have no effect on cEPO, which suggested that the N-glycans were not released (Figure 4B). This lack of complex sugars in N-glycans for proteins expressed in *P. pastoris* had also been reported for recombinant glycodelin A (Mukhopadhyay *et al.*, 2004) and equine herpesvirus glycoprotein D (Ruitenberg *et al.*, 2001).

The influence of deglycosylation on fractionated proteins was also examined. Figures 5 and 6 show that the molecular masses of all fractions of the secreted forms of both rhEPOs were reduced to a value corresponding to the predicted mass of the non-glycosylated polypeptide backbone shown in Figure 4. These results supported the suggestion that the variations in molecular mass (seen as a broad smear in western blots) were caused by differing contents of glycans. Note that the larger of the two bands observed for rhEPO-His (refer to Figure 4) only appeared from the third fraction onwards (Figure 5A), which suggests that earlier fractions did not contain the putative O-linked glycoisoforms.

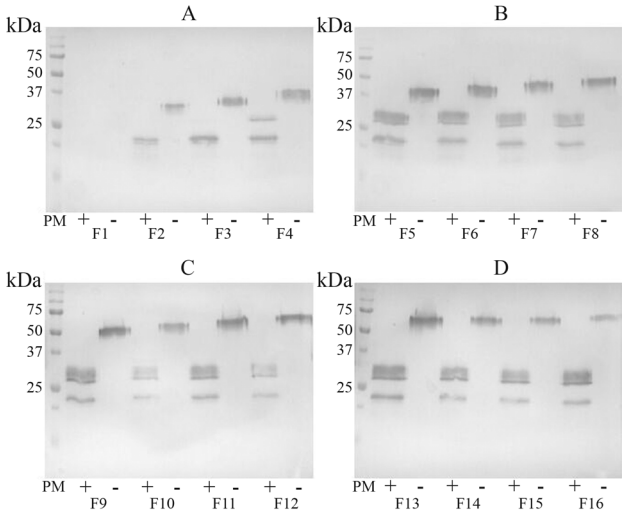

Figure 5 - Western blots of fractionated rhEPO-His after treatment with PNGase F (A, B, C and D). Lanes F1-12: samples after fractionation at 15 min intervals, PM – protein molecular mass markers, (+) treated with PNGase F, (-) not treated with PNGase F.

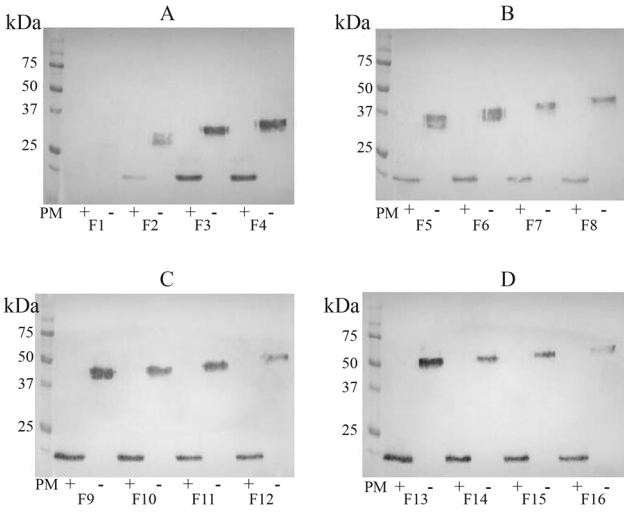

Figure 6 - Western blots of fractionated rhEPO-Stop after treatment with PNGase F (A, B, C and D). Lanes F1-12: samples after fractionation at 15 min intervals, PM – protein molecular mass markers, (+) treated with PNGase F, (-) not treated with PNGase F.

Analysis of recombinant protein activity

In mice injected with rhEPO-Stop and rhEPO-His the number of reticulocytes increased by 3.15 ± 0.63 and 3.03 ± 0.65, respectively (Figure 7). These increases were significantly ($p < 0.01$) higher than those observed in mice injected with PBS and the non-treated negative control. This increase in reticylocytes showed that the rhEPO-Stop and rhEPO-His produced in this study were functionally active despite variations in glycosylation when compared to native human EPO and CHO-expressed EPO.

Glycosylation is a common post-translational modification event in the secretory pathway of most eukaryotic expression systems; however, it is known to be species-, tissue- and cell type-specific (Brooks, 2006). Variable glycosylation is perhaps one reason for the variable molecular masses reported in different eukaryotic expression systems.

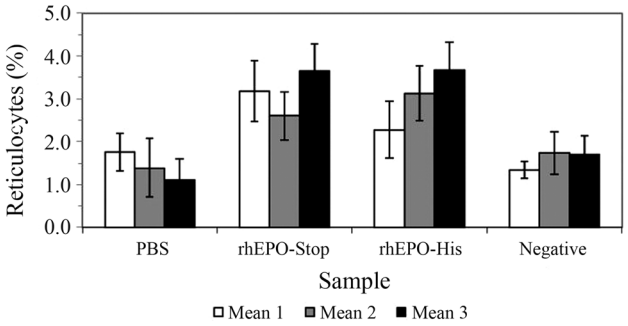

Figure 7 - Increase in the number of reticulocytes after multiple subcutaneous injections of rhEPO-Stop and rhEPO-His. The results are the mean ± SEM of five slides examined for each of three mice. The overall increase in the number of reticulocytes in mice (3/group) treated with rhEPO-Stop and rhEPO-His was 3.15 ± 0.63 and 3.03 ± 0.65, respectively. These percentages were significantly ($p < 0.01$) greater than observed in PBS-injected and negative control mice.

For example, recombinant EPO was ~35 kDa in CHO cells (Lin *et al.*, 1985), ~31 kDa in tobacco cells (Matsumoto *et al.*, 1995), ~25 kDa in *Drosophila* S2 cells (Kim *et al.*, 2005), > 29 kDa in *S. cerevisiae* (Elliott *et al.*, 1989) and ~30 kDa in *Physcomitrella patens* (Weise *et al.*, 2007), while human urinary EPO (or native EPO) is reportedly ~34 kDa (Dordal *et al.*, 1985). More recently, Celik *et al.* (2007) reported that the molecular mass of rHuEPO was 30 kDa and that the major glycan attached to all three N-linked glycosylation sites was $Man_{17}(GlcNAc)_2$. Curiously, other than for recombinant EPO produced commercially in CHO cells, none of the other studies mentioned above commented on the possible correlation or association between different rhEPO glycan profiles and the bioactivity of the protein. This aspect deserves further investigation.

Perhaps the most important issue to be addressed is whether proteins produced in yeast can be made to be more like the corresponding proteins in humans. Both EPOα and EPOβ, which have different isoform compositions, have been produced in mammalian cell systems (Storring *et al.*, 1998), and analyses of these EPOs have shown that in this case differences in their glycosylation patterns did not result in differences in immunogenicity (Hermeling *et al.*, 2003). Recombinant proteins produced in *P. pastoris* tend to be hypermannosylated. Although there are no detailed reports on the potential consequence(s) of hypermannosylation in the immune response of humans, hypermannosylation in *Pichia*-expressed recombinant proteins may be an important risk factor for increased immunogenicity.

Hypermannosylation in *P. pastoris* is initiated by the activity of α-1,6-mannosyltransferase (Och1p) (Dean, 1999). Inactivation of the *OCH1* gene, which leads to the elimination or minimization of N-linked glycosylation in *P. pastoris*, has been considered an important step in the "humanization" of these host cells (Bretthauer, 2003; Vervecken *et al.*, 2004). Other relevant "humanization" steps include the introduction of a gene that confers α1,2-mannosidase activity (to remove α1,2-mannose residues) and another gene (GlcNAc transferase I) that adds β1,2-linked GlcNAc residues to the α1,3-mannose arm. Engineered *P. pastoris* strains that can produce the so-called 'hybrid-type' N-glycan structures have been produced, with promising outcomes and minimal effect on yeast viability (Bretthauer, 2003; Choi *et al.*, 2003; Hamilton *et al.*, 2003; Vervecken *et al.*, 2004). More recently, Hamilton *et al.* (2006) reported extensive engineering of *P. pastoris* that also allowed the production of complex terminally sialylated glycoproteins, which is expected to increase the half-life and therapeutic potency of most glycoproteins. The availability of such yeast cell lines with `improved' protein products will significantly reduce the risk of problems associated with immunogenicity. This property, together with a reduction in the time and cost of production, may eliminate the need to use mammalian cells in the future.

Acknowledgments

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