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IMMUNO-AFFINITY EXTRACTION OF ERYTHROPOIETIN FROM HUMAN SERUM BY MAGNETIC BEADS

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Introduction

Background

Recombinant human erythropoietin (rhEPO) has been available as a drug since 1988. The gene for human erythropoietin (hEPO) was cloned in 1985 and subsequently introduced into different mammalian cell lines [1,2].

For several years athletes have been suspected to misuse rhEPO, especially in endurance sports. Until recently this was only rumours and speculations and there were no evidence of EPO abuse. By the discovery of rhEPO-ampoules in the care of cycle teams in Tour de France 1998 and also the admittance of EPO-misuse by several participants, there were strong indications that misuse of rhEPO may be prevalent within certain sports. One way to be able to control rhEPO abuse is to establish a method that directly can identify rhEPO in blood- and urine-samples taken during doping control.

Erythropoietin physiology

EPO is the principal regulator of erythrocyte production in mammals and is in adults mainly produced in the kidneys [3]. Tissue hypoxia is the main signal to the kidneys to start the synthesis of EPO, which is then secreted and subsequently stimulates the maturation of erythrocytes in the bone marrow [4]. EPO reacts with a specific receptor on its target cells. The expansion and maturation of the erythrocyte progenitor cells *in vivo* is accomplished in conjunction with other cytokine components, such as interleukin 3, granulocyte-macrofage-colony stimulating factor and the stem cell factor [5,6]. EPO can induce both proliferation and differentiation [7,8].

The molecular structure of human EPO

hEPO is synthesised as a 193 amino acid prohormone from which a 27 amino acid signal sequence is cleaved off in the cell. A carboxy-terminal arginine is lost from this 166 amino acid residue during passage into the circulation, leaving the mature hEPO hormone with 165 amino acids [9].

hEPO is heavily glycosylated with a carbohydrate moiety that constitutes about 40 % of the molecular weight [10-13]. It was purified to homogeneity from urine of patients with severe aplastic anaemia by Miyake et al. in 1977. The molecular mass of intact hEPO is ranging from 32-39 kDa, the polypeptide chain, however, is 18,2 kDa [10].

hEPO is glycosylated in four positions, with three N-linked oligosaccharides (Asn 24, Asn 38 and Asn 83) and one O-linked oligosaccharide coupled to serine 126. The acidic oligosaccharide structures of both urinary and recombinant hEPO contain mannose, fucose, N-acetylglucosamine, galactose, N-acetylgalactosamine and terminal N-neuraminic acid (sialic acid) [10-13].

hEpo has four cysteine residues that form two disulphide bridges where the binding between Cys 7 and Cys 161 is essential for the biological activity [10,14]. The N-linked oligosaccharides, both the poly-antennary structures and the terminal sialic acid residues, seem also to be necessary for the biological activity of hEPO *in vivo* [15,16].

Multiple forms of EPO

A glycoprotein consists of a collection of glycosylated forms that arise as a consequence of the biosynthetic pathways which develop the O-linked glycan chains and modify the N-linked oligosaccharide precursor. In the continuous process of posttranslational modifications that occur within the endoplasmatic reticulum and Golgi apparatus, the proteins simultaneously compete for the active sites of the glycosylation enzymes allowing variants or glycoforms to develop in a single protein population. Human urinary and rhEPO consist of one identical polypeptide-chain. However, they both contain different glycoforms due to microheterogeneity in their oligosaccharide moiety. A single batch of rhEPO may include numerous glycoforms [11,17-19]. The glycoform composition of EPO preparations varies with their source, differing between samples from serum and urine [20,21], between samples obtained from subjects under different pathophysiological conditions like anaemia, secondary polycythemia, polycythemia vera [18] and between urinary hEPOs (uhEPO) and different

rhEPOs [17,22]. In addition, the glycoform composition of an EPO preparation can be further influenced by the selectivity of the isolation procedures used to purify it [23].

Molecular differences between uhEPO and rhEPO

There are diverging reports concerning possible molecular differences between uhEPO and rhEPO. Several papers have indicated that rhEPO contain more basic glycoforms with a lower content of sialic acid residues than uhEPO [17,18,21]. A higher amount of tri-and tetra-antennary oligosaccharides in rhEPO than in uhEPO [11,24-26] has been suggested, but the opposite has also been indicated [17].

Materials and Methods

Materials

Anti-hEPO antisera and purified antibodies were purchased from Biogenesis, England (polyclonal rabbit antiserum); R&D Systems, England (rabbit IgG fraction); Sigma, USA (IgG fraction of antiserum developed in rabbit); Immunosys, Sweden (chicken antiserum); Santa Cruz Biotechnology Inc., USA (IgG fraction of antiserum developed against peptide sequence 35-57 of rhEPO); Biogenesis, England and Genzyme Diagnostics, USA (monoclonal mouse antibody IgG_{2a} (clone AE7A5) developed against the 26 amino acid peptide analogue of hEPO). Secondary antibodies conjugated with biotin were purchased from Sigma, USA and Pierce, USA and Streptavidin-horse radish peroxidase and ECL+ (a chemilumenescence substrate) from Amersham Pharmacia Biotech, Sweden. Dynabeads (tosylactivated M280 and M450) and magnets were provided from Dynal A.S, Norway. PhastSystem and Phastgels were from Amersham Pharmacia Biotech, Sweden.
Three standard rhEPO preparations were used in the studies; rhEPO from Boehringer-Mannheim, Germany, Eprex, a drug that in addition to Epoietin-α also contains human albumin (2.5 mg/ml) as stabilising protein, and Recormon, a drug containing different amino acids as stabilisers in addition to Epoietin-β.
Immobilon-P (polyvinylidene difluoride-PVDF) membranes (0.45 μm) acquired from Waters (MA, USA) were used in the immuno-blotting procedures. Electro-blotting was conducted in a semi-dry blotter from Ancos, Denmark. Recombinant peptide-N-glycosidase F (PNGase F) was provided from Oxford GlycoSciences, England.

Methods

SDS PAGE

Subsequent to reducing SDS polyacrylamide gel electrophoresis (PAGE) [27] in 12% gels on PhastSystem, the proteins were electro-blotted [28] onto PVDF membranes in a semi-dry blotting apparatus.

Immuno-blotting

After electro- or slot-blotting the membranes were incubated with different polyclonal antisera and developed with biotin-conjugated secondary antibodies including streptavidin labelled with horse radish peroxidase. ECL+ were used as chemilumenescence substrate.

Production of antigen-specific magnetic beads

Tosylactivated Dynabeads M280 and M450 were coated with different anti-EPO antibodies according to the manufacturers instructions [29]. The binding-efficiency and capacity of the beads were tested with iodinated (^{125}I) rhEPO (Boehringer-Mannheim).

Iodination of rhEPO

Pure rhEpo was labelled with ^{125}I to a specific activity of 75 μCi/μg. 2μg EPO was dissolved in 50 μl 0.05 M phosphate buffer, pH 7.5, and 0.75 μg Iodogen (Pierce, USA), corresponding to 0.2 mCi ^{125}I, was added.
After incubation for 5min. on ice, the mixture was loaded on a gelfiltration column (PD 10 containing Sephadex G-25 from Amersham Pharmacia Biotech) to separate ^{125}I rhEPO (tracer) from free iodine. Phosphate buffer with 0.2% human albumin was used as elution buffer.

Binding of ^{125}I rhEPO to hEPO-specific magnetic beads

In preliminary experiments, the optimal incubation time and temperature for the binding of EPO-tracer to anti-hEPO antibody no 5 (Table 1) covalently linked to magnetic beads were investigated (data not shown). At ambient temperature, maximal binding of ^{125}I rhEPO was obtained after 48 hours and these conditions were used in all subsequent experiments.
hEPO-specific magnetic beads were washed two times with phosphate (50mM)-buffered saline (PBS), pH 7.4, with 0.1% bovine serum albumin (BSA), and subsequently added

incubation-buffer to a final concentration of 0.1 mg/ml. The beads were incubated in either potassium buffer, pH 7.5 (50 mM KH_2PO_4, 50 mM KOH, 0.1 M NaCl, 8 mM NaN_3, 0.1% human serum albumin) with 0.25% normal mouse serum or PBS-BSA also with 0.25 % mouse serum. ^{125}I-labeled rhEPO was then added to each tube (approx. 10.000 counts per min.), and incubated under rotation for 48 hours at ambient temperature. After incubation, the tubes were placed on a magnet and the beads were washed three times with PBS-BSA and subsequently counted for 10 min. in a gamma counter.

Elution and detection of hEPO extracted by antigen-specific magnetic beads

From buffer: Buffer with standard rhEPO were incubated with washed beads (1 mg/ml) and incubated for 48 hours under rotation at ambient temperature. NaN_3 (0.02%) was added to prevent bacterial growth. After incubation, the beads were washed three times with PBS-BSA and then added freshly made SDS PAGE-sample buffer [27] included 2 % 2-mercaptoethanol. The amount of sample buffer was approx. 10 µl to 1 mg beads. The samples were boiled, placed in a magnet and the supernatant removed, and they were then ready to be run on reducing SDS PAGE.

From serum: After washing with PBS-BSA, hEPO-specific magnetic beads were incubated with human sera at a concentration of 0.1 mg beads/ml in potassium buffer with normal mouse serum. Patient-sera with various EPO-concentrations were used in the experiments. ^{125}I rhEPO was added to each tube and the samples were then incubated 48 hours under rotation at ambient temperature. NaN_3 (0.02%) was added as anti-microbial agent [29]. After incubation, the beads were washed three times with PBS-BSA by means of a magnet, and counted for 10 min. in a gamma counter.

Results

Test of different commercial antibodies against hEPO

Several different anti-hEPO antibodies (polyclonal-, monoclonal- and anti-EPO-peptide antisera/antibodies) from various species and manufacturers were tested with regard to specificity and activity against rhEPO standards (Table 1). Slot-blotting and SDS PAGE/ electro-blotting with subsequent immuno-staining were used to evaluate the different anti-hEPO antibodies (data not shown). In general, the antisera and antibodies showed low binding and cross reacted with several other proteins, also from human and different animal sera. Two polyclonal antisera against rhEPO (Table 1, n° 1 and 4) showed high activity in our systems, but cross reacted significantly with other proteins, e.g. human albumin and the molecular weight standards. An affinity-purified rabbit anti-hEPO antibody had acceptable activity, but gave a high background on immuno-blots (Table 1, n° 7). A monoclonal antibody (clone AE7A5) purchased from two different companies showed good specificity, but had low activity (Table 1, n° 2 and 3). An anti-hEPO-peptide antibody had very low activity, gave high background on the blots and cross reacted with human albumin (Table 1, n° 6). However, an affinity-purified polyclonal rabbit antibody produced against rhEPO had an acceptable binding and specificity (Table 1, n° 5). Two of the purified antibodies (Table 1, n° 2/3 and 5) were chosen for further experiments with magnetic beads.

Binding-experiments with hEPO-specific magnetic beads

Test of magnetic beads coated with different anti-hEPO antibodies.

Two anti-hEPO antibodies (Table 1, n° 2/3 and 5) were coated on tosylactivated magnetic beads (Table 2). The respective anti-hEPO-specific beads were then tested in their ability to bind ^{125}I rhEPO as a measure of their coupling-efficiencies. The best results were obtained with the purified rabbit antibody n° 5 (Table 1), and this antibody was used in the further experiments.

Test of different magnetic beads

Tosylactivated beads are provided in three different sizes (2.8 μm, 4.5 μm and 5 μm diameter particle-size). A batch of each of these beads was coated with antibody n° 5 (Table 1) and the binding of ^{125}I rhEPO were subsequently tested. The amount of antibody coupled per surface

Manufacturer	Antibody	Characteristics
N° 1. Biogenesis	Rabbit polyclonal anti-rhEPO antiserum	Acceptable activity. Strong CX against several proteins.
N° 2. Biogenesis **N° 3.** Genzyme Diagnostics	Mouse monoclonal affinity-purified IgG fraction	Low activity. High specificity.
N° 4. Immunosys	Chicken antiserum against rhEPO	Acceptable activity. Strong CX against several proteins.
N° 5. R&D Systems	Rabbit polyclonal anti-rhEPO affinity-purified IgG fraction	Low activity. High specificity.
N° 6. Santa Cruz Biotechnology	Goat polyclonal affinity-purified IgG fraction raised against aa-analogue 35-57 of hEPO	Low activity. Strong CX. High background on immuno-blots.
N° 7. Sigma	Rabbit polyclonal anti-rhEPO, affinity-purified IgG fraction	Acceptable activity. CX.

Table 1. Test of different commercial anti-hEPO antibodies; their manufacturers and properties (CX=cross-reactivity).

Magnetic beads	Antibody coated on the beads	ng antibody coupled per μm^2 beads	Cpm per μm^2 beads	Binding-efficiency, %
M280 (2.8μm)	Rabbit anti-EPO n° 5 (Table 1)	1.2	17.5	27
M280 (2.8μm)	Mouse anti-EPO n° 2/3	2.8	11.8	18
M450 (4.5μm)	Rabbit anti-EPO n° 5	3.9	37.4	37
M500 (5.0μm)	Rabbit anti-EPO n° 5	7.8	27.4	32

Table 2. Binding efficiancy of different anti-hEPO specific beads.

Magnetic beads in two sizes were coated with different anti-hEPO antibodies. Binding-efficiency was estimated with ^{125}I rhEPO.

area of the magnetic beads was calculated from the absorbance at 280 nm of the supernatant after coating, and the binding-efficiency was calculated based on the ^{125}I rhEPO activity coupled to the beads after incubation (Table 2). The hEPO-specific M280 beads had the smallest amount of bound antibody per μm^2 beads, while the antigen-specific M500 beads had the highest (Table 2). This was partly reflected in the amount of ^{125}I rhEPO that was coupled per surface area of the beads; the hEPO-specific M450 beads bound twice as much tracer as the M280 beads, while the hEPO-specific M500 beads gave a slightly poorer binding (Table 2). In our studies, the hEPO-specific M450 beads had a binding-efficiency of about 35 to 50 % compared to the M280 beads that bound 20 to 30 % of the added ^{125}I rhEPO, while M500 beads gave a binding in between. Table 2 shows the binding-studies demonstrating that the polyclonal rabbit antibody from R&D Systems coated on tosylactivated M450 Dynabeads gave the best results.

Determination of the capacity of the beads

A Scatchard plot [30] was made based on binding of ^{125}I rhEPO by M280 beads coated with anti-hEPO antibody n° 5 and displacement of the tracer by increasing amounts of standard rhEPO (Fig. 1). The calculated dissociation constant for the beads, Kd, was 1.45 x 10^{-11} mol/l

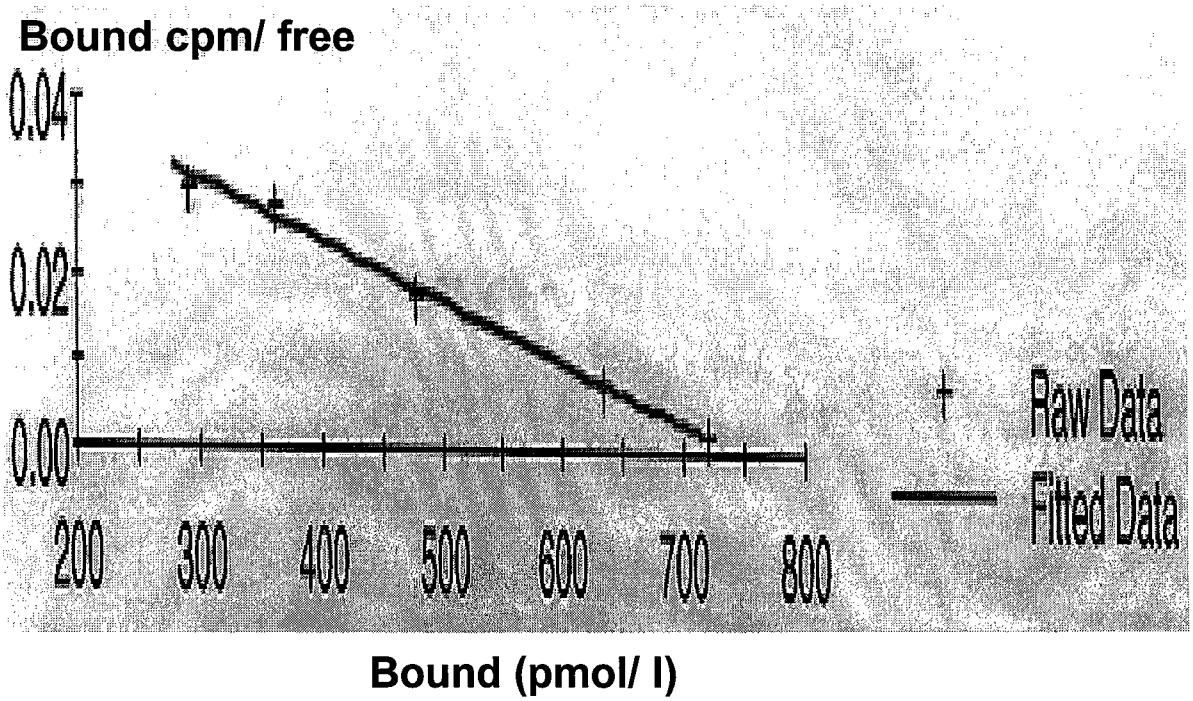

Figure 1. Scatchard plot [30] for the binding of ^{125}I rhEPO to M280 magnetic beads coated with rabbit anti-hEPO n° 5. The data represent the relation between bound and free (bound/free) ^{125}I rhEPO versus the tracer activity bound to the beads.

and the total amount of binding-sites was 7.46 x 10^{-10} mol/l. The capacity of the beads was 8.8 femtomol hEPO/ mg beads.

Immuno-magnetic extraction of human EPO

The hEPO-specific magnetic beads were used to extract EPO, both standard rhEPO (Fig. 2) from a buffered solution and endogenous EPO from human sera (Fig. 3). Different methods for the identification of bound hEPO were used in the two immuno-magnetic extractions. Standard rhEPO (Boehringer –Mannheim) was incubated in PBS-BSA with hEPO-specific magnetic beads for two days at room temperature. SDS PAGE and immuno-blotting were used to detect standard rhEPO extracted by the beads. Figure 2 shows an immuno-stained blot of different extracts from magnetic beads. In lane 1, 40 ng rhEPO (Boehringer-Mannheim) was loaded. In lane 3 and 4 controls were loaded, which were extracts from hEPO-specific magnetic beads treated like the other beads, but not added standard rhEPO. In both lane 5 and 6, immuno-stained protein-bands corresponding to the molecular size of standard rhEPO, were visualised. These lanes contained extracts from hEPO-specific beads incubated with 60 pmol of standard rhEPO.

In all lanes with bead-extracts, two higher molecular weight protein-bands could be seen, even more pronounced in the lanes without standard rhEPO added. These are probably IgG heavy chains leaking from the hEPO-specific beads due to the use of reducing conditions. These bands were shown to react strongly with anti-rabbit antibody in another application (data not shown).

The histogram in figure 3 demonstrates the binding of EPO from human sera to hEPO-specific magnetic beads and shows the displacement of ^{125}I rhEPO by increasing amounts of non-labelled rhEPO (Eprex). Human sera with three different concentrations of EPO (measured by an immunoassay from Nichols Institute Diagnostics), were incubated with the same amounts of iodinated EPO. The ability of the hEPO-specific magnetic beads to extract hEPO from serum was clearly demonstrated (Fig. 3).

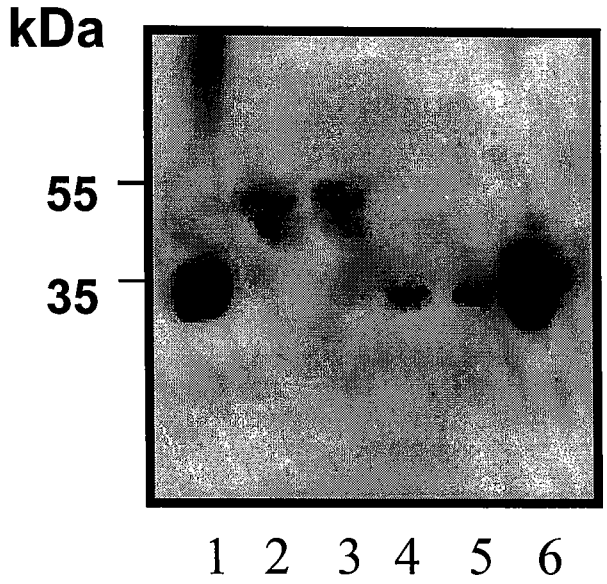

Figure 2. PVDF membrane after SDS PAGE with subsequent immuno-blotting of extracts from hEPO-specific magnetic beads. Lane 1: rhEPO, 40 ng. Lane 2 and 3: controls; extracts from beads without rhEPO. Lane 4 and 5: extracts from beads incubated with rhEPO (60 pmol). Lane 6: rhEPO, 60 ng. The membrane was developed with rabbit anti-hEPO n° 7 as the primary antibody.

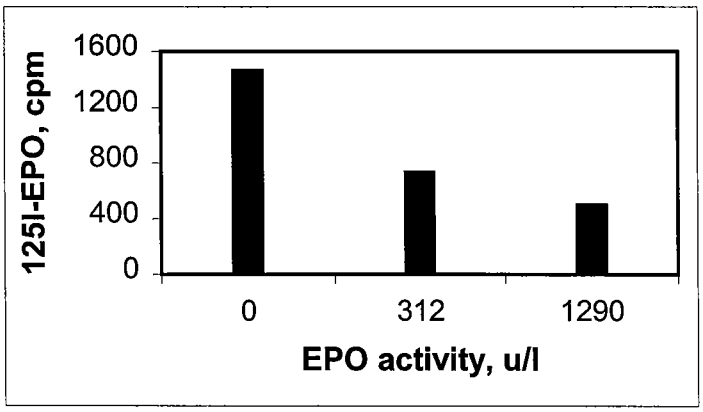

Figure 3. This histogram demonstrates binding of EPO from human sera to hEPO-specific magnetic beads, and shows the expected displacement of iodinated rhEPO by increasing amounts of non-labelled EPO. This demonstrates the ability of the magnetic beads coated with rabbit anti-hEPO n° 5 to extract hEPO from sera.

Discussion

In order to find an antibody against hEPO suitable for the use in immuno-affinity extraction, several commercial available anti-hEPO antisera and antibodies were tested (Table 1).
The quality of an immunoassay is dependent of both the specificity and affinity of the antigen/antibody-reaction. The standardisation of clinical assays for glycoprotein hormones, e.g. immunoassays, is difficult due to the extensive heterogeneity of these hormones (for review, see [31]). As a result of posttranslational modifications, glycoprotein hormones exist *in vivo* as an array of variants with differences in carbohydrate structure, isoelectric point (pI), biological activity and metabolic clearance (for review, see [31,32]). Recent findings show that the immunoreactivity of circulating hormone glycoforms may well account for observed variations depending on the antibodies used in the immunoassays. Thus, isoforms of glycoprotein hormones differ in their specific immunoreactivities as well as in their specific *in vivo* and *in vitro* bioactivities [31]. A specific antibody may have high affinity only to some of the glycoforms with lower affinity towards the others. Consequently, certain insecurity exists in what is really estimated with immunoassays when measuring glycoproteins. Some assays measure only the biological active forms (like in bioassays) and others the total content of isoforms [31,32].
We have successfully used tosylactivated magnetic beads as the solid phase in the immuno-magnetic extraction procedure (Fig. 2 and 3). By using magnetic beads as the solid phase, the method is simplified compared to more conventional procedures because the separation of the target antigen from other proteins in solution is easy. In addition, protein purification with magnetic beads is characterised by very low non-specific binding resulting in less background interference bands in SDS PAGE [33].
Our results showed that the binding-efficiency of the antibodies coated on the beads is dependent on the density of antibody molecules on each bead. If twice as much antibodies were coupled per μm^2 beads, the binding of ^{125}I rhEPO decreased significantly (data not shown). When the IgG molecules on the surface of the beads are coupled too close, binding of the antigen may be inhibited due to steric hindrance. In addition, the antibodies may not be able retain their correct conformations, and binding decreases. Also, with a higher concentration of antibody in the incubation-solution, the possibility of suboptimal orientation of IgGs on the beads may increase, and fewer molecules will have their antigen-binding sites available for the antigen. The covalent coupling to the beads may itself affect the

conformation of the IgG molecules and thereby the binding-properties of the antibody. M450 beads are nearly twice as large as M280, and as a consequence, the surface area is smaller than an equal amount of M280 beads. Despite this, M450 beads coated with anti-hEPO n° 5 (Table 1) provided a significantly better binding of rhEPO tracer than M280 hEPO-specific beads, probably due to the ability to bind more antibodies per μm^2 beads (Table 2). However, even if the M500 beads, coated with the same antibody, had nearly twice the amount of IgG per bead as M450, they bound less rhEPO tracer (Table 2). This may be a result from steric hindrance.

The normal EPO level in human serum is about 5 pmol per litre (10-20 U/l), and will probably rise at least twofold after repeated injections of rhEPO, depending on the dose [21,34,35]. The serum EPO-level has a circadian rhythm [36] and there are considerable intra- and inter-individual variations [18]. Therefore, to be able to detect rhEPO in serum or urine, a concentration step must be included. Immuno-magnetic extraction of hEPO (Fig. 2 and 3) was performed by the presently best available anti-hEPO antibody (Table 1, n° 5). Calculations from Scatchard plots demonstrated more hEPO binding sites on the M450 beads (data not shown) than the M280 beads coated with anti-hEPO antibody n° 5 (Fig. 1), which are consistent with our results from the studies of binding-efficiency (Table 2).

The association constant was estimated to be 6.9×10^{10} l/mol for the anti-hEPO M280 beads (Fig. 1), which is in an acceptable range of activity. However, due to the variable properties of this and the other commercial antibodies against hEPO that were tested, a considerable increase in the capacity of the beads may be gained by obtaining an antibody with a higher activity.

The M280 beads coated with anti-hEPO n° 5 (Table 1) had a capacity of approx. 10 femtomol per mg beads calculated from the Scatchard plot (Fig. 1). 1 mg beads are then theoretically able to extract a normal amount of EPO in 2 ml serum. By increasing the capacity of the beads, it will be possible to isolate adequate amounts of hEPO from a specified volume of serum or urine with the immuno-magnetic extraction procedure.

Before analysis by SDS PAGE, the isolated hEPO had to be eluted from the beads. By the employment of reducing SDS PAGE sample buffer [27], IgG heavy or light chains, or both, were probably cleaved off simultaneously. The immuno-stained protein bands in the lanes with extracts from hEPO-specific beads are probably corresponding to IgG heavy chains (Table 1). Reducing SDS sample buffer was used to avoid aggregation of EPO molecules, which had been observed during the studies of standard rhEPO preparations, and also to

ensure an optimal elution of bound EPO. The positive staining was probably a result of the biotinylated anti-rabbit IgG used in the development of the membrane, binding to the rabbit anti-hEPO primary antibody (Fig. 2). By excluding 2-mercaptoethanol in the sample buffer the problems with IgG leakage from the coated beads may be avoided.
The magnetic beads made specific to hEPO by covalent coating with rabbit anti-hEPO antibody n° 5 was demonstrated to be capable of extracting rhEPO standard from buffer (Fig. 2) as well as endogenous EPO from human serum (Fig. 3). The maximum binding of iodinated rhEPO observed was 50 % in buffer and 20 % in serum. The reason for the lower binding in serum, is at present unknown. It may be due to an inhibiting factor binding to either ^{125}I rhEPO or to the antibodies on the beads. The reduced binding may also be a result of degradation of the EPO molecule by iodine, which can disturb the structure of EPO due to its large size.

Conclusion

We have managed to extract both rhEPO from buffered solution and endogenous EPO from human serum by the use of tosylactivated magnetic beads as the solid phase in an immuno-magnetic extraction procedure. The rabbit anti-hEPO antibody n° 5 was chosen because it was supplied as an affinity-purified IgG fraction, had an acceptable specificity and gave a very low unspecific background on immuno-blots. The best results were obtained when this antibody was coated onto M450 beads. However, as already mentioned, to be able to increase the capacity of the beads, the technique has to be refined with regard to other, more active antibodies against hEPO.

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