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Polyclonal antibodies aimed to differentiate EPO and NESP

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Abstract

Antibodies play a key role in the detection of erythropoietin (EPO). There is an obvious interest in developing antibodies able to differentiate between EPO and NESP (the so called New Erythropoiesis Stimulating Protein or darbepoetin alfa) thus increasing the selectivity of the current method used.

Three peptides 15-20 amino acid (aa) in length corresponding to the sequence containing differences between EPO and NESP were synthesized: pepEPO (aa 81-95 of EPO :LVNSSQ**PW**E**P**LQLHVC-NH_2), pepNESP1 (aa 81-95 of NESP: LVNSSQ**VN**E**T**LQLHVC-NH_2) and pepNESP2 (aa 86-104 of NESP: Q**VN**E**T**LQLHVDKAVSGLRSC-NH_2).

After coupling to keyhole limpet hemocyanin (KLH) they were used to immunize rabbits following standard protocols. The obtained sera were first characterized by enzyme-linked immunosorbent assay (ELISA) using the synthetic peptide-OVA conjugates and then purified by affinity chromatography. The corresponding preparations of purified antibodies were assessed by ELISA, SDS-PAGE and isoelectric focusing (IEF) followed by western blot. Experiments were also performed in parallel using some commercial anti-EPO antibodies in order to compare their selectivity.

Specificity analysis indicated that anti-pepEPO antibodies recognized rEPO but did not react with NESP. No specific antibodies were detected in the sera from rabbits immunized with the pepNESP1-KLH conjugate whereas the anti-pepNESP2 antisera reacted with NESP but was also able to recognize rEPO although with lower intensity. After reduction and partial de-glycosylation an increase in sensitivity was seen for all antibodies obtained.

These results indicate that the anti-pepEPO antibodies fully discriminate rEPO from NESP, and, under certain conditions, anti-pepNESP2 was also able to partially discriminate NESP from rEPO. For all the obtained polyclonal antibodies further studies in order to identify the reactivity against uEPO must be performed, as well as to establish the best conditions for their use in the standard techniques employed to confirm the rEPO and NESP misuse in sports.

Introduction

Polyclonal and monoclonal antibodies (Ab) are essential tools for the detection of EPO. The current doping control method for the detection and identification of recombinant EPO (rEPO) and NESP in urine [1] uses a monoclonal antibody (mAb) as the primary antibody for detection in western blot. This Ab (clone AE7A5) was raised against a peptide corresponding to the sequence of the first 26 N-terminal amino acids of EPO. Regarding its specificity, this Ab recognizes rEPO as well as urinary EPO (uEPO) and NESP with similar affinities. Other monoclonal and polyclonal Ab have been developed against peptides corresponding to regions remote from glycosylation sites in order to ensure accessibility, maximizing recognition of the native proteins [2]. In other cases, those raised against whole rEPO were checked to neutralize the biological activity or be insensitive to changes in glycosylation [3,4]. However by using those approach no differences could be expected nor were found in their affinity for rEPO, uEPO or NESP.

It is well established that small synthetic peptides (15-20 AA length) can be used to raise Ab able to recognize EPO and analogues. Since EPO and NESP do contain differences in the amino acid sequence [5], it should be possible to choose the appropriate antigen containing those differences in order to generate Ab able to discriminate between them. On the other hand, those differences are directly related with glycosylated regions which may potentially hinder Ab interaction. Furthermore, differences in glycosylation between rEPO, uEPO and NESP may also play a role in such discrimination.

The aim of the present work was the development of rabbit polyclonal antibodies against EPO and NESP using specific synthetic peptides containing the differing protein sequence. Those antibodies will be characterized for their ability to specifically discriminate between EPO and NESP under different immunological conditions and techniques including those currently employed in doping control.

Materials and methods

Peptide synthesis and coupling.

Three peptides were synthesized: pepEPO (aa 81-95 of EPO), pepNESP1 (aa 81-95 of NESP) and pepNESP2 (aa 86-104) [6]. Besides, a cysteine residue was added to all of them as the handle for the final linkage to an immunogenic carrier protein. Peptides were synthesized by the solid-phase method using the Fmoc/^{t}Bu protection. HPLC purified peptides were coupled to keyhole limpet hemocyanin (KLH) with a 3-maleimidobenzoic acid N-hydroxysuccinimide ester (MBS) bridge and to ovalbumin (OVA) with a glutaraldehyde bridge, through the terminal cysteine residues added.

Immunization schedule.

Two rabbits were immunized with 200 μg of each synthetic peptide-KLH conjugate in MPL-TDM adjuvant (Sigma) every two weeks. After four immunizations, they were boosted monthly. Animals were bled from the auricular artery 7 days after each immunization starting from the third. Serum samples were stored frozen at –40 ºC until they used.

Enzyme-linked Immunosorbent Assays (ELISA).

ELISA assays were performed on 96-well plates (MaxiSorp, Nunc) in order to titer the antisera and check their ability to recognize the different peptides and proteins.

Well plates were coated with the different antigens (peptide-ovalbumin conjugate, rEPO or NESP) by addition of 50 μl of a 10 μg/ml solution in PBS and incubation at 37 ºC for 1 hour. Unbound sites were blocked with 1% gelatin in PBS for 30 min. Anti-sera were diluted in PBS containing 0.1 % (v/v) Tween 20 (PBST), added to the antigen coated wells and incubated for 1 hour at 37 ºC. After washing with PBST, 50 μl of a secondary antibody solution (Alkaline phosphatase-conjugated swine anti-rabbit immunoglobulin, Dako) 1/1000 diluted in PBST were added and incubated at 37 ºC for 1 hour. After washing with PBST, 50 μl of a 4-methyllumbelliferyl-phosphate (4-MUP, Sigma) solution at 1 mg/ml in triethanolamine buffer (pH 9.5) were added. Fluorescence was measured at 460 nm in a Cytofluor^{TM} 2350 Fluorescence Measurement System (Millipore). Pre-immune rabbit sera and a commercially available polyclonal anti-EPO antibody from Sigma (ref. E0271) were used as negative and positive controls, respectively. In some cases, rEPO and NESP were also reduced and alkylated before coating.

Immunoaffinity chromatography.

Anti-peptide antibodies were purified by affinity chromatography against its corresponding immobilized peptide. In each case, 5 mg of peptide were coupled to 5 ml of EAH Sepharose 4B (Amersham Biosciences) using MBS as bridge. Columns were then end-capped with 2-mercaptoethanol.

Antisera were diluted 1/3 in 10 mM Tris-HCl pH 7.6 and loaded onto the column for 2 h. Then, the column was washed with 10 mM Tris-HCl, 0.5 M NaCl pH 7.6. Bound antibodies were eluted with 100 mM glycine-HCl pH 2.5. Fractions were immediately neutralized with 1 M Tris and kept frozen at –20 ºC until they were used. Immunoaffinity columns were stored at 4 ºC in 10 mM Tris-HCl pH 7.6 with 0.01 % NaN_3.

SDS-PAGE and western blot.

rEPO and NESP were applied in 10 % SDS-PAGE gels (run for 1 hour at 100 V) and transferred onto nitrocellulose membranes (Protran, Schleicher and Schuell) at 400 mA for 1.5 hours. In some cases, immobilized glycoproteins were partially deglycosilated incubating the membrane with 10 mM $NaIO_4$ in 25 mM sodium acetate buffer pH 4.5 for 1 h at room temperature in the dark [7]. Non-specific binding sites were blocked using 1 % BSA in PBS-T. Immunoblotting was performed by incubating the primary anti-EPO and anti-NESP antibodies for 90 min, antirabbit –biotin (Dako) as a secondary antibody for 1h and finally streptavidine-HRP (Zymed) for 45 min. All the incubations were made in PBS containing 1 % BSA and between incubations three washes in PBS-T were made. Finally, reactions were developed using ECL reagent (Amersham). The image was produced using auto-radiographic film.

IEF and double blot

Isoelectric focusing (IEF) analyses were performed as described by F. Lasne at al. [1] with the adaptations to the present study as follows: samples (20 µl) were applied to an isoelectric focusing (IEF) gel with a pH range 2 to 6 ((T=5 %, C=3 %, 7 M urea). After separation (1 W/cm until 3600 Vh at 8ºC) the proteins were electroblotted to a poliyvinyliden difluoride (PVDF) membrane (30 min at 0.8 mA/cm^2) using a basic transfer buffer (25 mM Tris-192 mM glycine). The membrane was directly incubated with the primary antibody solution or after pre-treatment with 10 mM $NaIO_4$ in 25 mM sodium acetate buffer pH 4.5 in order to produce partial deglicosyltation. The purified anti-EPO (81-95) and anti-NESP (86-104)

antibodies were diluted (1/35) with PBS-Tween 20 (0.1 %) and incubated overnight at room temperature. In the case of the polyclonal anti-EPO antibody from Sigma (ref. E0271) it was used at a 1/500 dilution with PBS-milk (1 %) and incubated for one hour. The monoclonal anti-human EPO antibody (clone 9C21D11, R&D Systems) was evaluated at different dilutions (1/1000, 1/500, 1/400, 1/200 and 1/100) and was incubated for 1 hour in all cases. The monoclonal anti-human EPO antibody (clone AE7A5, R&D Systems), used in routine doping control, was used as a reference control under the conditions already described elsewhere [1]. After washing, only the membranes incubated with the antibody from Sigma and the monoclonal antibodies were subjected to the double blotting procedure. The antibodies were thus transferred by electro-blotting (0.8 mA/cm^2, 30 min) to a second PVDF membrane using an acidic transfer buffer (0.7% acetic acid).

In accordance with the origin of the primary antibody, the secondary antibody used was the anti-rabbit polyclonal antibody labeled with biotin (1/3000, Dako) or the anti-mouse polyclonal antibody labeled with biotin (1/4000,). In both cases, the antibody was incubated for 1 hour at room temperature. Finally, the membrane was incubated with Streptavidin-horseradish peroxidase (Biospa, SPA) for one hour.

The chemiluminiscence light was produced by the addition of the peroxidase substrate (Super signal west femto stable peroxide from Pierce). The light was detected using a CCD camera (LAS-1000, Fujifilm).

Results and Discussion

Selection of the antigen

Peptides corresponding to the region containing differences in amino acid sequence between EPO and NESP were chosen as antigens (Figure 1) in an attempt to maximize the selectivity of the antibodies obtained. Three peptides were finally synthesized. Their sequences are shown in the Table 1. Sera from animals immunized with the pepNESP1-KLH conjugate did not elicited antibody response, hence a second peptide (pepNESP2) was also synthesized. This new peptide was longer and has its differing amino acids in the C-terminal region, making them more exposed for immunological recognition.

EPO

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  1  APPRLICDSR VLERYLLEAK EAENITTGCA EHCSLNENIT VPDTKVNFYA
 51  WKRMEVGQQA VEVWQGLALL SEAVLRGQAL LVNSSQPWEP LQLHVDKAVS
101  GLRSLTTLLR ALGAQKEAIS PPDAASAAPL RTITADTFRK LFRVYSNFLR
151  GKLKLYTGEA CRTGDR
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NESP

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  1  APPRLICDSR VLERYLLEAK EAENITTGCN ETCSLNENIT VPDTKVNFYA
 51  WKRMEVGQQA VEVWQGLALL SEAVLRGQAL LVNSSQVNET LQLHVDKAVS
101  GLRSLTTLLR ALGAQKEAIS PPDAASAAPL RTITADTFRK LFRVYSNFLR
151  GKLKLYTGEA CRTGDR
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Figure 1. Amino acid sequence of EPO and NESP. Differing amino acids are indicated in bold.

Table 1. Sequence of the peptides selected as antigens for the production of antibodies against rEPO and NESP. All peptides were added a cysteine residue to be used for coupling to form the final immunogen.

ACRONYM	AMINO ACID SEQUENCE	LOCATION	Nº AA
pepEPO	LVNSSQ**PW**E**P**LQLHVC-NH_2	81-95	15
pepNESP1	LVNSSQ**VN**E**T**LQLHVC-NH_2	81-95	15
pepNESP2	Q**VN**E**T**LQLHVDKAVSGLRSC-NH_2	86-104	19

Titration of the antisera

Post-immunization antisera were first tested for specific antibodies using ELISA tests prepared for the purpose in which well plates were coated with the synthetic peptides used for immunization.

Results (Figure 2) showed that antisera anti-pepEPO reacts with the pepEPO peptide, in much lower extent with the pepNESP1 and hardly recognised pepNESP2. By contrast, antisera from pepNESP2-immunized rabbits recognized pepNESP2 , in a lower extent pepNESP1 and hardly recognised pepEPO.

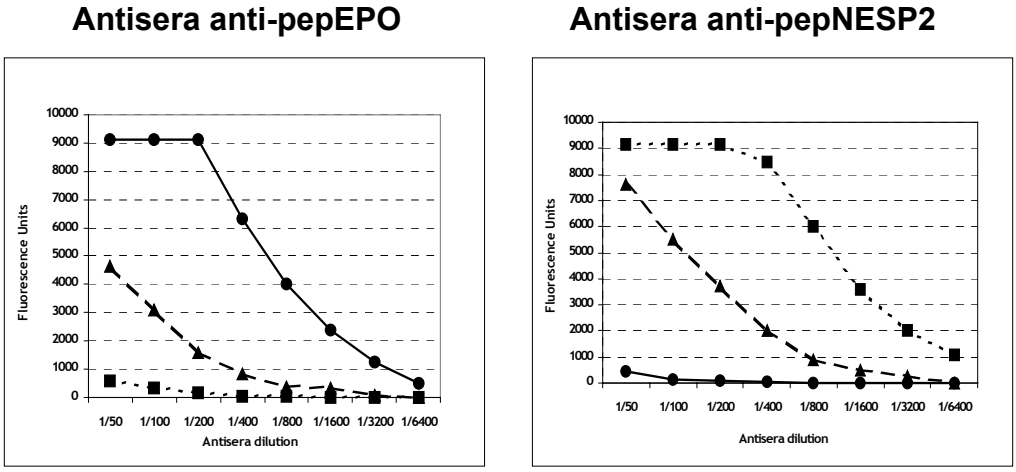

Figure 2. Results obtained in the titration of the antisera against the peptides used for immunization. ●—● reaction with pepEPO. ▲– –▲ reaction with pepNESP1. ■- - -■ reaction with pepNESP2.

Purification of the antisera by affinity chromatography

Specific anti-peptide antibodies were purified against the immunized peptide coupled to sepharose. Eluted antibodies were further tested by ELISA against rEPO and NESP native and reduced and alkylated entire proteins. The results obtained (Figure 3) show that the antibodies raised against pepEPO were also able to recognize the entire protein while they did not react with NESP. The affinity for EPO greatly increased after reduction and alkylation indicating a better exposure of the recognized sequence after de-naturation. As for the corresponding peptides, the antibodies raised against pepEPO did not recognize NESP.

Regarding the antibodies raised against pepNESP2, they were able to recognize NESP but were also able to recognize EPO with lesser affinity. Surprisingly, while the affinity for EPO increased after reduction and alkylation it did not in the case of NESP. This surprising result needs further confirmation.

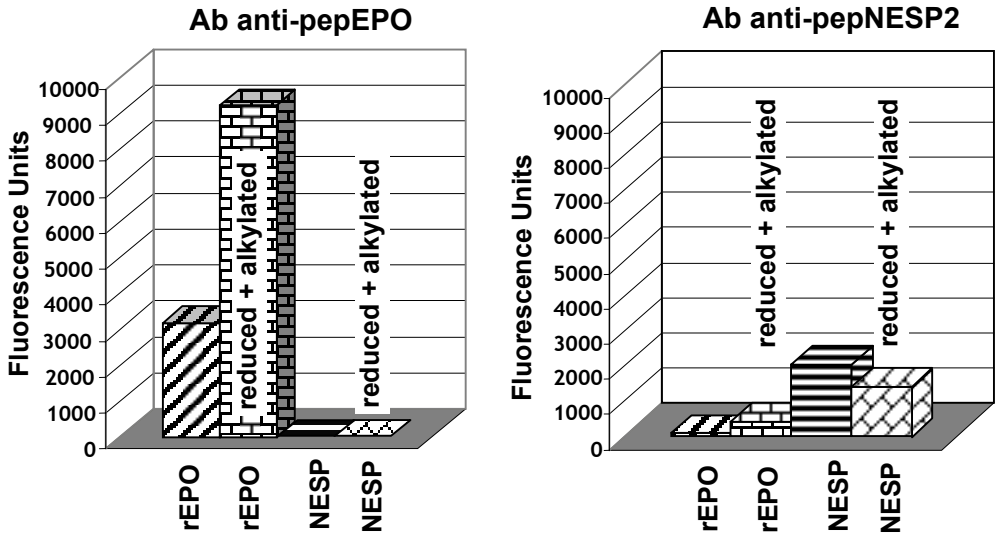

Figure 3. Results obtained in the titration of the purified antibodies against rEPO and NESP (entire proteins) as such and reduced-alkylated.

SDS-PAGE and Western Blot analysis

Purified antibodies were assayed against the whole rEPO and NESP molecules by SDS-PAGE electrophoresis and Western blot. Those experiments were also carried with a commercial polyclonal antibody against EPO from Sigma (product nº E0271) for comparison reasons.

Results (figure 4) show that anti-NESP purified antibodies recognizes both molecules with the same pattern that the Sigma antibody, whereas the polyclonal anti-EPO reacts specifically with the rEPO molecule.

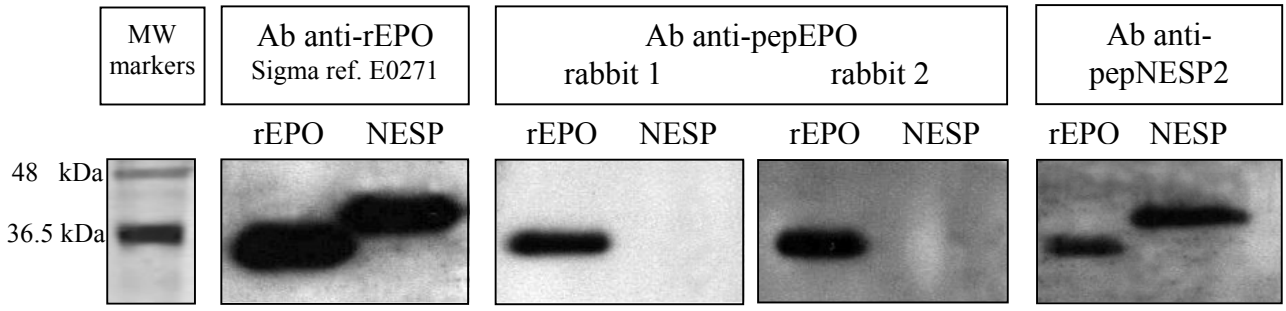

Figure 4. Recognition of rEPO and NESP by different antibodies after separation by SDS-PAGE.

In order to have an idea of the limit of detection that could be reached using the current preparations of purified antibodies, gels were prepared with decreasing concentrations of rEPO and NESP. In order to maximize sensitivity, proteins were reduced and also submitted to a treatment with sodium periodate. Results obtained are shown in Figure 5.

Results indicate that the $NaIO_4$ –treatment enhances the detection suggesting that the carbohydrate chains are partially masking the epitope recognized by the antibodies.

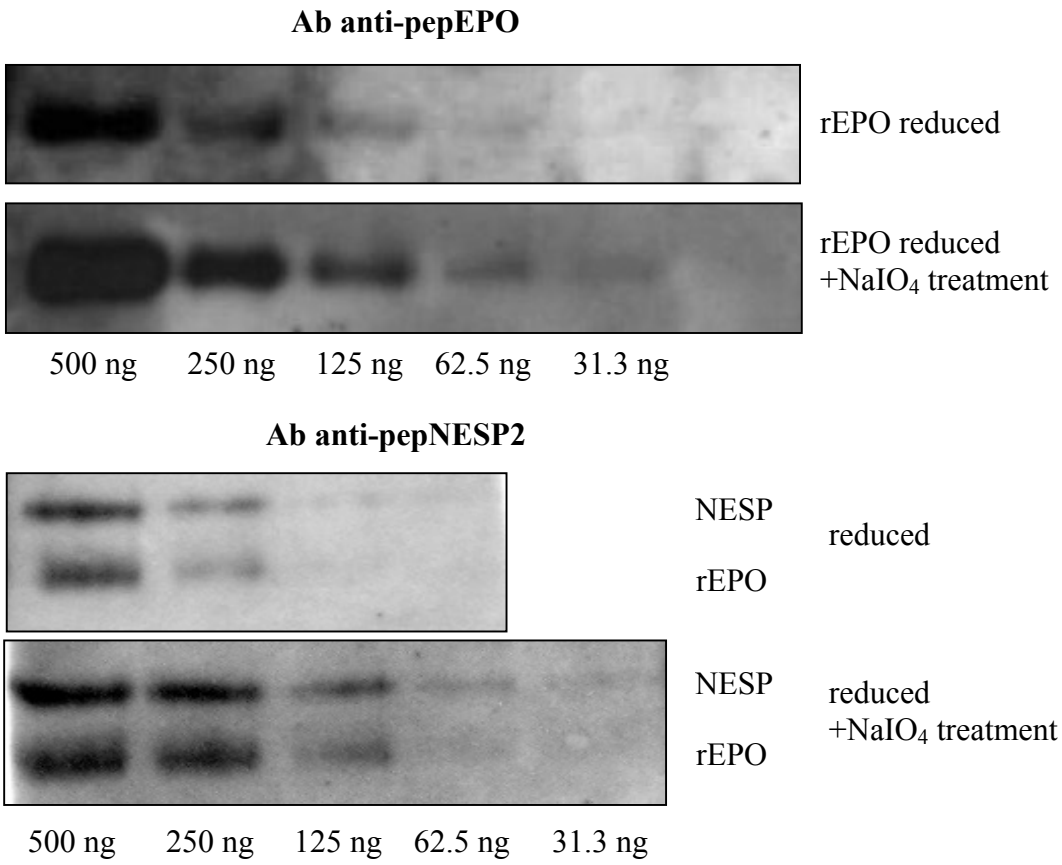

Figure 5. Limit of detection for rEPO and NESP reached by SD-PAGE using the different antibody preparations obtained and under different treatments.

IEF recognition

The specificity of the polyclonal antibodies under the current IEF method used in doping control was also evaluated. Results are shown in Figure 6.

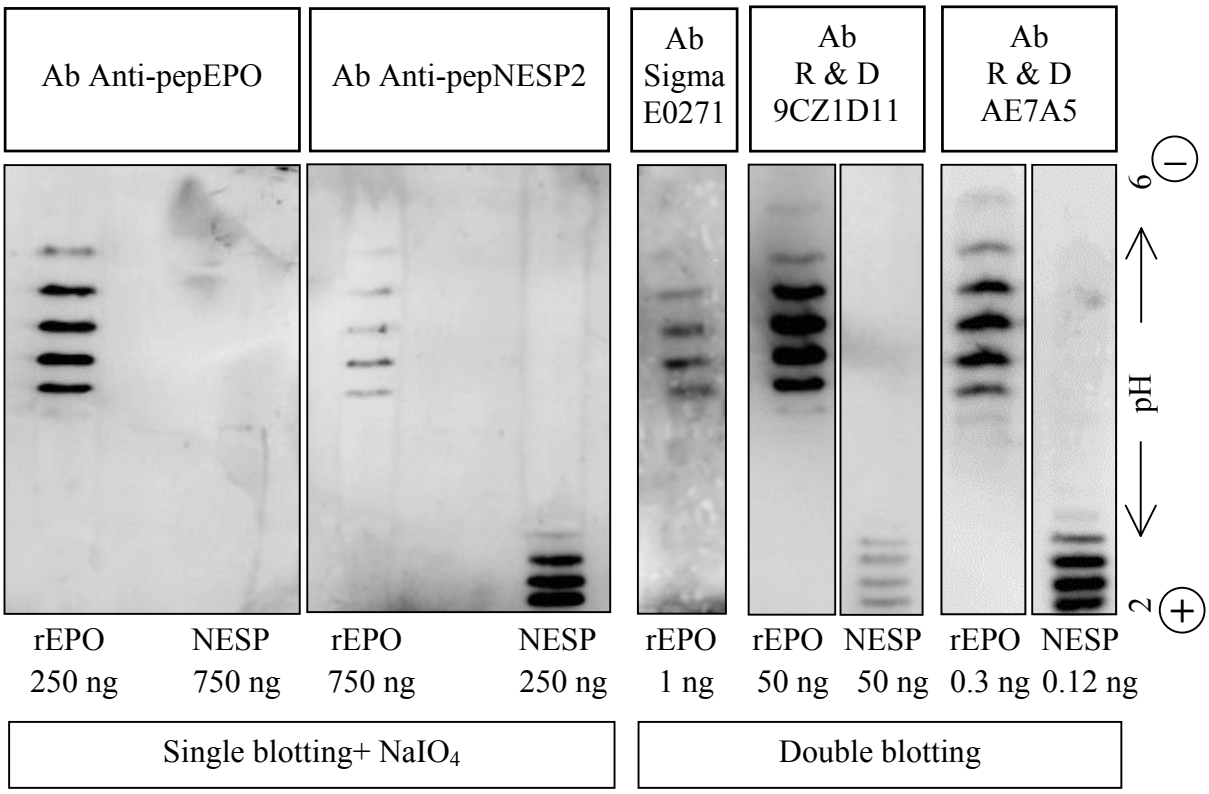

Figure 6. Comparison of the analysis of rEPO and NESP by IEF using different primary antibodies and conditions.

Under IEF conditions, the antibody against pepEPO still showed its specificity for rEPO while NESP was not detected at all. Interestingly, the antibody against pepNESP2, although was able to detect both substances, showed a much higher sensitivity for NESP than for rEPO that it showed under SDS-PAGE conditions. This may have particular relevance for doping control. The monoclonal antibody from R&D routinely used for doping control as primary antibody (clone AE7A5) showed similar response for rEPO and NESP while another clone (9CZ1D11) showed greater affinity for rEPO than for NESP.

Regarding sensitivity, the preparation from clone AE7A5 has shown to be the most sensitive one. Other monoclonal antibodies from the same manufacturer (e.g. clone 9CZ1D11 from

R&D) showed sensitivity in the order of 100 times lower particularly for NESP. The comparison with another commercial polyclonal antibody (the one from Sigma) also showed a reduced sensitivity. Using the preparations of purified polyclonal antibodies obtained in this work sensitivity was still lower. Further concentration and purification strategies, particularly for those fractions with the highest affinity will be necessary in order to improve such sensitivity.

Further efforts have to be also addressed to see if discrimination between endogenous urinary EPO and recombinant EPO can be achieved. The production of the corresponding monoclonal antibodies, essential in order to guarantee reproducible and sustainable results, is out of the scope of the present work.

Conclusions

- Synthetic peptides of 15-20 amino acids can be used to produce antibodies with differing affinities for rEPO and NESP.
- An in-membrane treatment with sodium periodate showed to improve detection.
- The affinity of the antibodies and thus their specificity may vary depending on the technique: ELISA, SDS-PAGE, IEF.
- Other commercially available antibodies also result in differing selectivities.
- The Mab from clone AE7A5 has shown to be the gold standard regarding sensitivity.
- The use of those antibodies for the selective purification of biological matrices continues being of major importance for the structural elucidation of EPO glycoforms.

Acknowledgement

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