ANNEXE 7

Publications scientifiques F. Lasne relatives à l'EPO

Recombinant erythropoietin in urine

An artificial hormone taken to boost athletic performance can now be detected.

Erythropoietin is a hormone that stimulates the production of new red blood cells (erythropoiesis). Although athletes use recombinant human erythropoietin illicitly to boost the delivery of oxygen to the tissues and enhance their performance in endurance sports, this widespread doping practice cannot be controlled in the absence of a reliable analytical procedure to monitor it. Here we describe a new technique for detecting this drug in urine following its recent administration.

The stimulation of erythropoiesis by erythropoietin (EPO) makes this drug very attractive to sportspeople wishing to improve their aerobic power, although the International Olympic Committee banned its misuse ten years ago. Detection has been a problem — analysis of haematological[1] or biochemical[2] parameters indicates only that erythropoiesis has been stimulated, but cannot confirm that drug administration is to blame.

To detect administered hormone directly means that exogenous, recombinant EPO must be differentiated from natural, endogenous EPO. A promising electrophoretic method[3] has proved impractical for screening by the antidoping laboratories. We have developed an analytical procedure for detecting recombinant EPO in urine and have applied it to specimens from cyclists participating in the the infamous Tour de France 1998 competition, which was sullied by scandals about EPO doping.

Owing to microheterogeneity in their structures, natural and recombinant EPO comprise several isoforms, some of which have charge differences and can be separated by isoelectric focusing (Fig. 1). We found that the isoelectric patterns of the two recombinant EPO-α and -β forms are very similar (both have an isoelectric point, pI, in the range 4.42–5.11); although EPO-β has an extra basic band, both differ from natural, purified urinary EPO, which has more acidic bands (pI 3.92–4.42), probably due to post-translational modifications such as glycosylation, which is species- and tissue-type-dependent[4]. Such differences in the urine analysis allowed us to ascribe excreted EPO to a natural or recombinant origin.

We developed an immunoblotting procedure to obtain a reliable image of EPO patterns in urine. Our results (Fig. 1) indicated that the patterns from control subjects consisted of about 10 bands of pI 3.77–4.70, in accord with the purified natural urinary EPO pattern, whereas those from subjects treated with recombinant EPO contained more basic bands, reflecting the presence of recombinant isoforms, and sometimes acidic bands as well, depending on the presence of endogenous isoforms. The presence of exogenous hormone was always evident: any individual injected with recombinant EPO showed a striking transformation of their initial EPO urine pattern.

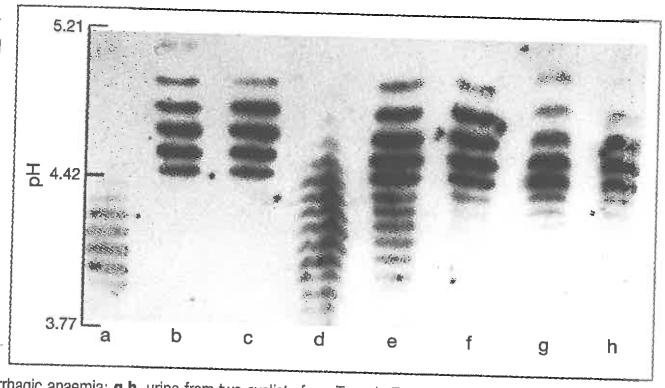

Figure 1 Autoradiograph of isoelectric patterns of exogenous and endogenous erythropoietin (EPO). Images were obtained by chemiluminescent immunodetection of blotted EPO after isoelectric focusing. **a,** Purified commercial human urinary natural EPO (Sigma); **b,** recombinant EPO-β (Neorecormon, France); **c,** recombinant EPO-α (Eprex, France); **d,** urine from a control subject; **e,f,** urine from two patients treated with Neorecormon EPO for post-haemorrhagic anaemia; **g,h,** urine from two cyclists from Tour de France 1998 (samples concentrated by ultrafiltration). Note the 'mixed' appearance of the pattern in **e**. The cathode is at the top; pH values are indicated on the left.

We assayed 102 frozen urine samples from participants in the Tour de France 1998 cycling competition for EPO by using an enzyme-linked immunosorbent assay. Twenty-eight of these samples had EPO levels above the normal range of 0–3.7 international units per litre (mean, 0.48 IU per litre, $n=103$; 77 samples were below the minimum detectable concentration of 0.6 IU per litre). We analysed the 14 samples presenting with the highest concentrations (7–20 IU per litre): although characterization of the EPO source does not require such high levels for urine analysis, we selected these samples for isoelectric focusing as they were more likely to contain exogenous hormone; indeed, they all gave rise to a banding pattern typical of recombinant hormone.

Our method for detecting recent exposure to recombinant EPO in athletes could be useful for in-competition controls in events of long duration (for example, cyclists have been known to use exogenous EPO continuously for 6 months at a time), but should find its principal application in out-of-competition testing.

Françoise Lasne, Jacques de Ceaurriz
National Anti-Doping Laboratory,
92290 Châtenay-Malabry, France

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Double-blotting: a solution to the problem of non-specific binding of secondary antibodies in immunoblotting procedures

Françoise Lasne *

Laboratoire National de Dépistage du Dopage, 143, avenue Roger Salengro, 92290 Châtenay-Malabry, France

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Abstract

"Double-blotting" (DB) was developed to overcome the problem of non-specific binding of secondary antibodies in immunoblotting (IB). After it had been probed by the primary antibody, the membrane with the blotted proteins was assembled with a second blank membrane and submitted to a second blotting under acidic conditions. The primary antibody molecules were thus desorbed from their corresponding antigen and transferred onto the second membrane, whereas the antigen and the interfering proteins remained bound to the first one. The second membrane could then be probed by the secondary antibodies without the risk of non-specific binding. This method was developed for the study of erythropoietin (EPO) in concentrated urine since a strong non-specific binding of biotinylated secondary antibodies to some urinary proteins had been observed using classical IB protocols.

Keywords: Double-blotting; Immunoblotting; False positives; Secondary antibodies; Erythropoietin

1. Introduction

Since blotting techniques were first applied to proteins (Erlich et al., 1979; Renart et al., 1979; Towbin et al., 1979), the immunological detection of a protein immobilized on a synthetic membrane support (IB), has become a widely used method (Towbin and Gordon, 1984). From the same basic procedure many protocols have been developed (Bers and Garfin, 1985; Garfin and Bers, 1989). Indeed, various problems may arise when developing a new IB procedure and require the modification of a protocol suitable for some other application. In particular, the non-specific binding of the antibodies used gives rise to false positives. In response to such a difficulty, a new method called "double-blotting" was developed to overcome the problem of non-specific binding of secondary antibodies.

2. Materials and methods

2.1. Samples

Physiological human urine samples (proteinuria < 0.15 g/l) were concentrated 50 times for dot-blot experiments and 500 to 1000 times for isoelectric

Abbreviations: PBS, phosphate-buffered saline; BSA, bovine serum albumin; EPO, erythropoietin; rHuEPO, recombinant human erythropoietin; PVDF, polyvinylidene fluoride; IB, immunoblotting; DB, double-blotting

* Tel.: +33-1-4660-2869; fax: +33-1-4660-3017.
E-mail address: f.lasne@wanadoo.fr (F. Lasne).

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focusing (IEF) experiments, by ultra filtration using Centricon and Centricon plus 20 from Millipore (MW cut-off 30,000 Da).

For dot-blot experiments, serial two-fold dilutions of recombinant human EPO (rHuEPO) (Boehringer) were prepared over the concentration range 15,600 to 120 IU/l (129.5 to 1 μg/l) in either 0.1% (w/v) bovine serum albumin (BSA) (Sigma) in phosphate-buffered saline (PBS) pH 7.4 or in concentrated urine.

For IEF experiments, the retentates of ultra-filtered urine were adjusted to an EPO level of 1500 IU/l using 50 mM Tris buffer pH 7.3. Pharmaceutical rHuEPO (Janssen-Cilag) was diluted to 2000 and 1000 IU/l using 0.1% BSA–50 mM Tris buffer pH 7.3.

2.2. Dot-blotting

Immobilon P (Millipore) polyvinylidene fluoride membranes (PVDF) having a 0.45 μm pore size were wetted in methanol, rinsed in water and equilibrated in PBS according to the instructions of the manufacturer. The samples (3 μl) were then applied.

2.3. Isoelectric focusing and electro-blotting

The retentates of ultra-filtration and the dilutions of rHuEPO (20 μl) were electrophoresed in a polyacrylamide gel ($T = 5\%$, $C = 3\%$), pH gradient 2–6 (ampholytes 2–4 and 4–6 from Serva), 7 M urea. The gel was then submitted to a classical semi-dry electro-blotting process in 25 mM Tris, 192 mM Glycine buffer at 0.8 mA/cm^2 for 30 min using the same membrane as in dot-blotting and a Multiphor II NovaBlot unit (Amersham Pharmacia Biotech).

2.4. Classical IB of EPO

Following the dot or electro-blotting, the membranes were blocked using 5% non-fat milk (Regilait France) in PBS for 1 h at room temperature. The blocked membranes were then incubated with the primary antibody solution, namely 1 μg/ml monoclonal mouse anti-human EPO (clone AE7A5) (R&D) in 1% non-fat milk PBS, for 1 h at room temperature and washed in a 0.5% non-fat milk PBS wash solution (six changes).

The membranes were then incubated with the secondary antibody solution: 0.25 μg/ml biotinylated goat anti-mouse IgG (H + L) (2813 CE from Valbiotech) in 1% non-fat milk PBS, for 18 h at 4°C.

The other tested secondary antibodies included: biotinylated rabbit anti-mouse immunoglobulins E354 (Dako), biotinylated F(ab')$_2$ fragment of rabbit anti-mouse immunoglobulins E0413 (Dako) and goat anti-mouse IgG (Fc specific) peroxidase conjugate A-0168 (Sigma).

Washing was then performed using three changes of wash solution for 3 × 5 min and the membranes (except in the case of A-0168 conjugate) were incubated in a streptavidin:biotinylated peroxidase complex solution (SPA from Valbiotech) (peroxidase concentration 1.25 μg/ml) for 1 h at room temperature.

After washing with three changes of PBS (3 × 5 min), the membranes were incubated with a chemiluminescent substrate Covalight (Covalab) according to the manufacturer's instructions. Detection of luminescence was performed either by autoradiography using Hyperfilm-ECL (Amersham–Pharmacia) or exposure in a Luminescent Image Analyzer LAS-1000 Plus CCD camera (Fuji film).

2.5. Double-blotting (DB)

Following the dot or electro-blotting, the membranes were processed as described above for blocking, incubation with the primary antibody and washing of the classical IB.

The principle of the DB was to transfer the primary antibody separately from the blot membrane to a new support called the DB membrane.

Two variants of DB were successively developed. The first one (pressure DB) gave good results with the dot-blot experiments and the second one (electro DB) proved to be more efficient in the case of the IEF experiments.

2.6. Pressure DB

A second PVDF membrane (DB membrane) was cut to the dimensions of the blot membrane, wetted in methanol, rinsed in water and equilibrated in 0.1 M glycine (Sigma)/HCl pH 2.5. This DB membrane was layered on a 3-mm filter paper sheet (Whatman)

wetted with the same buffer and laid on three dry filter paper sheets. The blot membrane was layered onto the DB membrane and covered with a filter paper sheet wetted with the same buffer. This assembly was placed between two glass plates and secured by two clamps as shown by Fig. 1A. The contact was maintained for 5 min.

2.7. Electro DB

The DB membrane was conditioned in a 0.7% (v/v) acetic acid solution. It was then layered onto the blot membrane between two stacks of filter paper wetted with the same solution in the semi-dry electrophoretic blotting instrument with the blot membrane and the DB membrane facing the anode and the cathode, respectively (Fig. 1B). A constant intensity of 0.8 mA/cm^2 was applied for 10 min.

The primary antibody was thus desorbed from its corresponding antigen and transferred onto the DB membrane (Fig. 1C). The membranes were then disassembled and the DB membrane was quenched in 5% non-fat milk PBS for 1 h at room temperature and then successively incubated with the secondary antibody, the strepavidin–peroxidase complexes and the chemiluminescent substrate as described above for classical IB of EPO.

In order to demonstrate the efficiency of the transfer, the blot membranes after the DB process (pressure and electro DB) were retained and submitted to probing by either the primary, or directly by the secondary antibody as described above for classical IB of EPO.

3. Results

Classical IB of rHuEPO serially diluted in 0.1% BSA–PBS showed a detection sensitivity of 5.9 mIU (about 49 pg) in dot-blot experiments (Fig. 2A).

When the same experiment was carried out using serial dilutions of rHuEPO in concentrated human urine, a quite different result was observed with the same intensity in the different spots, regardless of the EPO concentration (Fig. 2B). This demonstrated that the signal was not specific for EPO, but related to the presence of urinary proteins.

The same non-specific results were obtained when the primary antibody was omitted in the protocol (Fig. 2C), whereas no signal was obtained when the secondary antibody was omitted. Therefore, the reagent responsible for the false positives was the secondary biotinylated anti-mouse IgG. This non-specific binding of the secondary antibody was very strong and was not inhibited by changes in buffer pH and ionic strength. It also occurred in the presence of 0.05% Tween-20 or 4 M urea.

Different other secondary antibodies were tested: biotinylated rabbit anti-mouse immunoglobulins), biotinylated $F(ab')_2$ fragment of rabbit anti-mouse immunoglobulins and goat anti-mouse IgG peroxi-

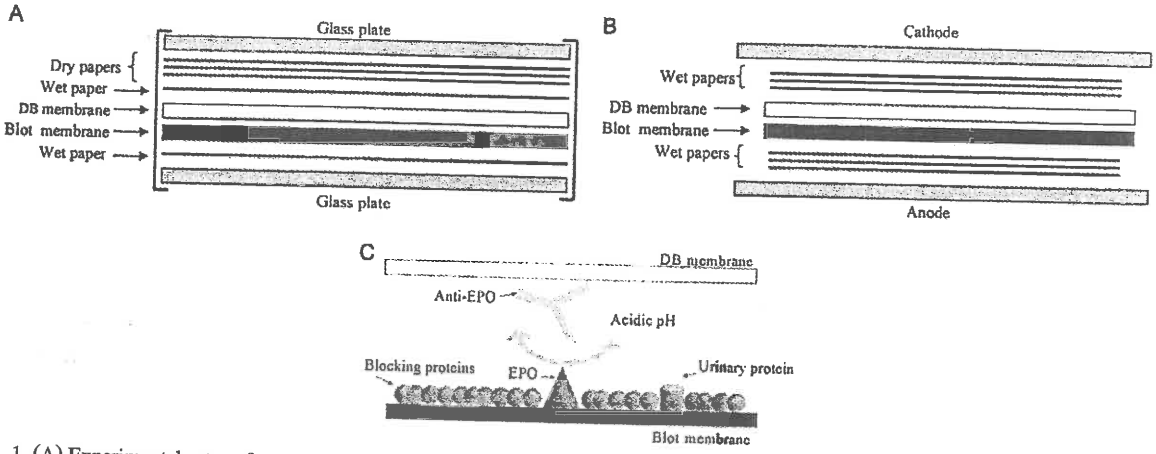

Fig. 1. (A) Experimental set-up for pressure double-blotting (DB). (B) Experimental set-up for electro-DB. (C) Principle of double-blotting.

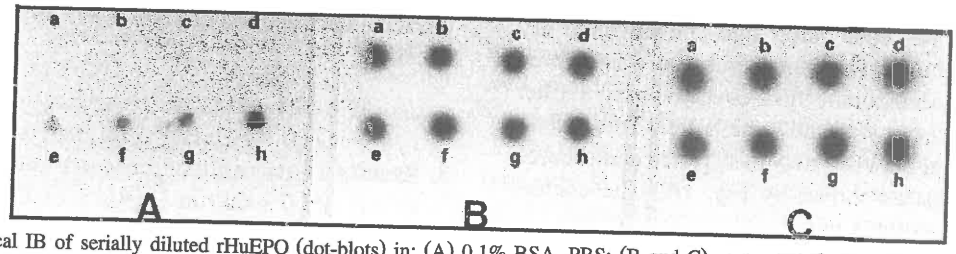

Fig. 2. Classical IB of serially diluted rHuEPO (dot-blots) in: (A) 0.1% BSA–PBS; (B and C) concentrated urine. The primary antibody (anti EPO) was omitted in (C). rHuEPO quantities applied: (a) 0.37 mIU (3.07 pg); (b) 0.73 mIU (6.06 pg); (c) 1.46 mIU (12.1 pg); (d) 2.93 mIU (24.3 pg); (e) 5.86 mIU (48.6 pg); (f) 11.7 mIU (97.1 pg); (g) 23.4 mIU (194.2 pg); (h) 46.9 mIU (389.3 pg).

dase conjugate, without any improvement in the results (data not shown).

Only the DB protocol proved to be efficient. Indeed, when the serial dilutions of rHuEPO in concentrated urine were tested using DB an increasing signal corresponding to the concentrations of EPO appeared on the DB membrane (Fig. 3B′). The non-specific signal observed in classical blotting (Fig. 2B) was recovered on the blot membrane (Fig. 3B) as shown by probing it with the secondary antibody after the DB. In contrast to the primary antibody, the urinary proteins were thus retained on the blot membrane during the DB. Therefore, this method separated the specific signals (on the DB membrane) from the non-specific signals (on the blot membrane).

Using dilutions of rHuEPO in 0.1% BSA–PBS, it was possible to compare the sensitivities of classical IB with this new process: the same image was obtained on the IB (Fig. 2A) and DB (Fig. 3A′) membranes. Thus, the DB process did not induce any loss of sensitivity.

Probing the blot membrane with the secondary antibody after the DB did not give rise to any signal, indicating the complete transfer of the primary antibody from the blot to the DB membrane (Fig. 3A).

The signal obtained on the DB membrane was indeed specific for EPO since it did not appear when the blot membrane was not incubated with the primary antibody before the DB (Fig. 3C′).

When the blot membrane of rHuEPO in 0.1% BSA–PBS was probed again with the primary antibody after the DB, the image of the increasing concentrations of EPO was recovered (Fig. 4). This demonstrated that EPO was retained on the blot membrane and that its immunological properties were preserved.

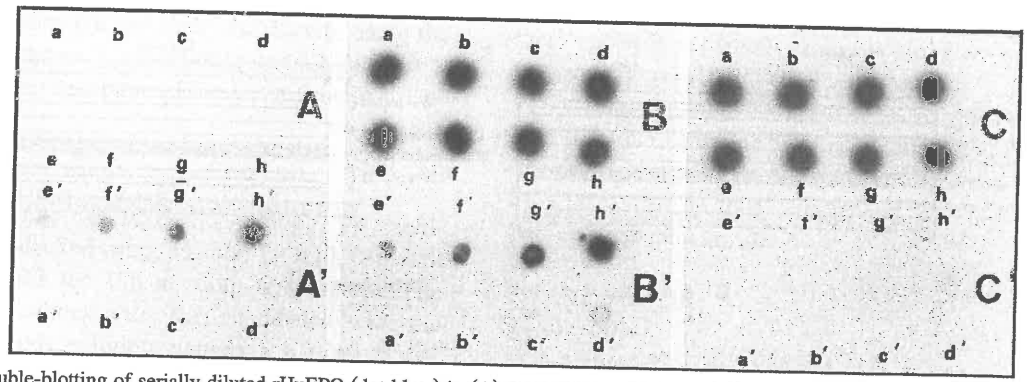

Fig. 3. Double-blotting of serially diluted rHuEPO (dot-blots) in (A) 0.1% BSA–PBS; (B and C) concentrated urine. The primary antibody (anti EPO) was omitted in (C). The same quantities of rHuEPO as in Fig. 2 were applied from (a) to (h). (A′), (B′) and (C′) are the DB membranes corresponding to the blot membranes (A), (B) and (C), respectively. Since for DB, the face of the blot membrane with the applied samples was in contact with the DB membrane, the disposition of (a′) to (h′) spots is the mirror image of that corresponding to a to h spots. The blot membranes (A), (B) and (C) were probed with the secondary antibody after the DB process.

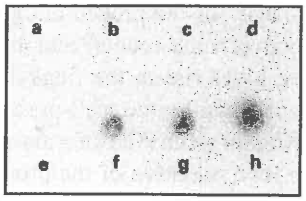

Fig. 4. Re-probing of the dot-blot membrane with anti-human EPO after DB. The same quantities of rHuEPO as in Fig. 2 were applied from (a) to (h).

Classical IB after isoelectric focusing of the two different dilutions of rHuEPO in 0.1% BSA–Tris gave rise to an isoelectric pattern composed of five bands located in a p*I* range of 4.4–5.1 (Fig. 5A).

When electro DB was performed after IEF of the same rHuEPO dilutions, similar patterns were recovered on the DB membrane (Fig. 5B).

The luminescence of the five isoforms (designated as 1 to 5 from the cathode to the anode) was evaluated using a CCD camera and showed that the DB process did not induce any significant change in the distribution of their relative intensities (Table 1).

When concentrated urine samples were submitted to classical IB following IEF, a non-specific isoelectric pattern was observed, corresponding to the binding of the secondary antibody on some of the urinary proteins focusing in this pH interval. Therefore, it was not possible to distinguish the bands corresponding to the endogenous EPO (Fig. 6A).

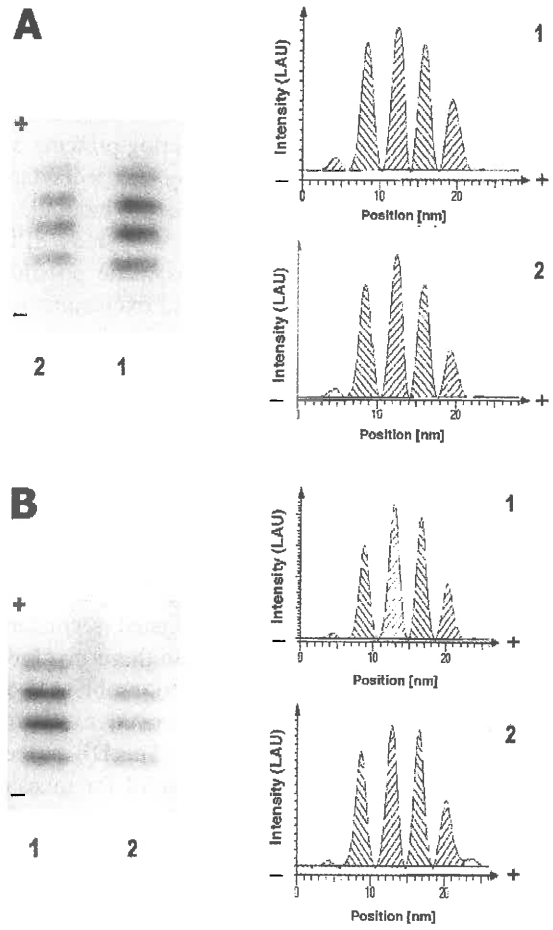

Fig. 5. Isoelectric patterns of rHuEPO diluted in 0.1% BSA–Tris. (A) classical IB; (B) DB. rHuEPO quantities applied: (1) 40 mIU (332 pg); (2) 20 mIU (166 pg). The intensity of the different isoforms is expressed in Linear Arbitrary Units (LAU). The positions of cathode (−) and anode (+) are indicated on the images and the corresponding profiles.

Table 1
Distribution of the luminescence intensity among the five isoforms of rHuEPO using IB and DB processes. (a) 2000 IU/l; (b) 1000 IU/l

Isoform no.	1	2	3	4	5
IB					
a	2.3%	26.5%	30.6%	24.7%	15.9%
b	1.9%	27.3%	35.0%	24.6%	11.2%
DB					
a	1.9%	29.6%	33.3%	23.0%	12.1%
b	0.7%	29.8%	30.9%	25.1%	13.5%

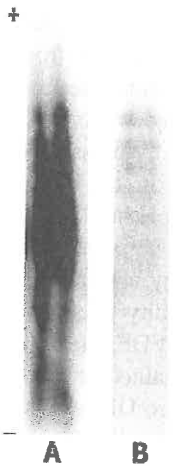

Fig. 6. Isoelectric patterns of concentrated urinary endogenous EPO. (A) classical IB; (B) DB. The positions of cathode and anode are indicated by − an + respectively.

Using the electro DB process, it was possible to reveal specifically the isoelectric pattern of the endogenous EPO on the DB membrane (Fig. 6B). This pattern differed from that of recombinant EPO with more acidic isoforms in the p*I* range 3.9–4.4.

4. Discussion

Non-specific interactions between blotted proteins and unrelated secondary antibodies generate false positives in immunoblotting techniques. Gershoni (1988) has emphasized the difficulty of resolving such a problem. Indeed, the nature of these interactions is not fully understood. In some cases, they appear to be hydrophobic since they are reduced by the presence of detergents. However, in other cases the detergents are ineffective.

Moreover, the use of Tween-20 alone as a blocking agent has been reported to cause false positives (Bird et al., 1988). Nevertheless, the final suggestion of Gershoni concerning this problem is, when possible, to use different probes.

In our experiments the false positives observed with immunoblotting of EPO in concentrated urine were related to the non-specific binding of the secondary biotinylated goat anti-mouse IgG antibodies to some of the blotted urinary proteins. In contrast, it did not occur in blotting experiments with BSA. All attempts to prevent or reduce this non-specific binding were ineffective when working directly on the blot membrane. It was then decided to transfer the primary antibody molecules from the blot membrane onto a separate membrane before performing the problematic probing with the secondary antibody. This was achieved by pressure or electro transfer in acidic conditions from the blot membrane.

Decreasing the pH, a classical desorption method in immuno-affinity chromatography, induces dissociation of the primary antibody from the blotted antigen. Since the acidity does not affect hydrophobic interactions with PVDF, the antigen and the interfering proteins are retained on the blot membrane. Use of pressure or electro DB results in the capture of the desorbed antibody by the facing membrane without any diffusion.

A specific image of the probed antigen can thus be obtained. It should be noted that the result is actually an "image" of the probed antigen since it is only the antibody on the second membrane and not the antigen that gives rise to the final signal.

However, as shown by the well-preserved proportions of the different rHuEPO isoforms after DB, this image is quite representative of the probed antigen.

The process is not time consuming, 5–10 min are sufficient to ensure an efficient transfer of the primary antibody onto the DB membrane. Since only the primary antibody leaves the blot membrane, the latter can be probed again with a primary antibody.

Although in our experiments this was tested using the same primary antibody after DB, different antibodies could probably be used to detect their corresponding antigens. Thus, DB appears as an efficient method to strip the primary antibody from a blotting membrane in view of the fact that it could be readily re-probed. However, its essential value is in isolating the primary antibody from the interfering proteins on a membrane that is then probed by the secondary antibody without any risk of non-specific binding.

This procedure has been developed to study EPO isoelectric patterns in urine and has made possible the differentiation of endogenous and exogenous recombinant hormone for anti-doping control purposes (Lasne and de Ceaurriz, 2000). However, it may be useful in other unrelated studies involving the non-specific binding of the secondary antibody as it often occurs when working with samples containing very high levels of protein. The procedure has been tested by immunoblotting transthyretin after isoelectric focusing of human serum. Although the samples (serum) and the immunological reagents (polyclonal primary antibodies and gold-conjugated secondary antibodies), were quite different from those involved in the EPO experiments, the same problem was observed and the DB procedure proved to be an efficient solution (data not shown). The DB procedure is expected to be especially useful for investigating proteins that are present in minute amounts in complex biological media.

Acknowledgements

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Detection of isoelectric profiles of erythropoietin in urine: differentiation of natural and administered recombinant hormones

Françoise Lasne,* Laurent Martin, Nathalie Crepin, and Jacques de Ceaurriz

Laboratoire National de Dépistage du Dopage, 143 Avenue Roger Salengro, 92290 Châtenay-Malabry, France

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Abstract

Erythropoietin (EPO) is normally present in urine at a low concentration (about 1 IU/L, i.e., about 10 ng/L) for a total protein concentration of at least 50 mg/L. A method to study the isoelectric profile of this hormone from 20-ml urine aliquots without previous purification was developed. This method involves isoelectric focusing of the retentate from ultrafiltered urine. Both the ultrafiltration and the isoelectric focusing required precautionary measures to prevent EPO degradation by the proteases that are present in urine. Because classical immunoblotting gave rise to an unspecific detection of various urinary proteins in the focused retentate, it was essential to use the "double-blotting" process developed to solve this problem. Sufficient sensitivity was achieved using amplified chemiluminiscent detection after the blotting membrane was treated with dithiotreitol. The patterns that were revealed from various urinary samples proved to be highly heterogeneous as they were composed of more than 10 isoforms in a p*I* range of 3.7–4.7. Clear transformation of the patterns was observed in the case of treatment by the recombinant hormone, suggesting that this method can be regarded an efficient tool for indicating recombinant EPO misuse in sports. It may also open new investigations in the field of physiologic or pathologic exploration.

Erythropoietin (EPO)[1] is a glycoprotein hormone produced by the kidney in adult humans. It stimulates red blood cell production by promoting the proliferation and differentiation of erythroid progenitor cells. Since 1985, recombinant human EPO (rHuEPO) has been available for therapeutic use in certain forms of anemia [1]. This hormone, however, quickly became misused as a doping agent for endurance athletes to improve aerobic performances, and the International Olympic Committee officially prohibited it in 1990.

Wide et al. [2,3] reported a lower negative median charge of rHuEPO in comparison with the natural hormone. In their studies, they used zone electrophoresis, at pH 8.6, of serum and urine in agarose suspension, with subsequent determination of the EPO concentration in the different fractions eluted from the electrophoretic column. These authors proposed this method for antidoping control but, because of considerable practical difficulties, it has never been applied in antidoping laboratories.

It is well known that both the natural and the recombinant form of EPO present extensive microheterogeneity in relation to posttranslational modifications in proteic moiety. Many investigations have focused on the glycosylation of this hormone since it is particularly developed and substantial with respect to its biological properties [4]. All studies have demonstrated that glycosylation is substantially implicated in the hormone's microheterogeneity [5,6]. Other modifications that have not yet been clearly investigated in the case of EPO, however, may also contribute to this heterogeneity. Some of these posttranslational events are influenced by the nature and the environmental conditions of the cell that produces the

* Corresponding author. Fax: +33-146-603-017.
E-mail address: f.lasne@lndd.com (F. Lasne).

[1] *Abbreviations used:* EPO, erythropoietin; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; MWCO, molecular weight cutoff; ELISA, enzyme-linked immunosorbent assay; IEF, isoelectric focusing; BSA, bovine serum albumin; DB, double-blotting; DTT, dithiotreitol.

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protein. Since human natural and recombinant EPO are synthesized in human kidney and Chinese hamster ovary (CHO) cells, respectively, some of these modifications may be different in the two hormones. In so far as these modifications affect their electrical charge, the resulting molecules can be separated into isoforms by appropriate techniques. Differences in their isoelectric profiles thus seemed to be a potential means to differentiate between natural and recombinant EPO. We report here a method that was developed to investigate the isoelectric profiles of this hormone in urine.

Materials and methods

Urine samples

Urine samples were obtained from healthy controls and rHuEPO-treated volunteers at different postinjection times during an administration trial (subcutaneous injections of Eprex 4000 from Janssen–Cilag at decreasing doses from 50 to 20 IU/kg, three times per week for 7 weeks). The details of this trial will be published at a later date. All the samples were kept frozen at −20 °C until they were analyzed.

Reagents

The recombinant EPO was from Janssen–Cilag (France) as Eprex for Epoetin α, from Roche as NeoRecormon for Epoetin β, and from Amgen as Aranesp for Darbepoetin α. Protease-free Tris and glycine were from Acros Organics, NaCl was from Panreac, and sucrose was from USB. Ampholytes Servalyt 2–4, 4–6, and 6–8 were from Serva. Phosphate-buffered saline (PBS) (0.01 M sodium and potassium phosphate buffer, pH 7.4, containing 2.7 mM potassium chloride and 0.137 M sodium chloride), dithiothreitol (DTT), and *N*-acetyl-D-glucosamine were from Sigma. Purified Tween 80 was from Pierce. The protease inhibitor cocktail, Complete, and pepstatin were from Roche. Steriflip microfiltration (0.22 μm) units, Centricon-plus 20, and Centricon YM 30 ultrafiltration (molecular weight cutoff (MWCO) 30,000 Da) units, and Durapore (0.65 μm) and Immobilon-P (0.45 μm) membranes were from Millipore. Urea Plus one and wheat germ lectin Sepharose 6MB (WGA Sepharose) were from Amersham Biosciences. The enzyme-linked immunosorbent assay (ELISA) for human EPO Quantikine IVD and monoclonal mouse anti-human EPO (AE7A5) were from R&D. Biotin-labeled purified goat antibodies to mouse IgG were from P.A.R.I.S (France). Streptavidin:biotinylated peroxidase complexes were from Biospa (Italy), nonfat dry milk was from Régilait (France), and chemiluminescent substrate Covalight was from Covalab (France).

Ultrafiltration of urine

Urine was kept frozen at −20 °C until it was prepared. After thawing at room temperature, 2 ml of 3.75 M Tris/HCl, pH 7.4, and 0.4 ml of Complete solution (1 tablet in 2 ml of water) were added to 20 ml of urine. After centrifugation at 2700 RCF and 20 °C for 10 min, the supernatant was microfiltered under vacuum through a 0.22-μm Steriflip device. This filtrate was then submitted to a first ultrafiltration in a Centricon Plus-20 (MWCO 30,000 Da) by centrifugation at 3570 RCF and 20 °C for 20 min. The retentate was then washed with 20 ml of 50 mM Tris/HCl, pH 7.4, and 0.4 ml of Complete solution in the same Centricon Plus-20 by centrifugation under the same conditions. The washed retentate (about 100–200 μl) was then recovered as indicated by the manufacturer, transferred to a Centricon YM30 having the same MWCO, and further ultrafiltered by centrifugation at 2340 RCF and 20 °C for 1 h to obtain a final volume of 20–80 μl. The final retentate was assayed for its EPO level by ELISA and was kept frozen at −20 °C until isoelectric focusing (IEF). In some experiments, an additional step to reduce the protein content of the final retentate was included in the preparative protocol. In this case, the retentate from the Centricon YM30 device was adjusted to a volume of 400 μl with 50 mM Tris/HCl, pH 7.4, containing 0.2 M NaCl and incubated with an equivalent volume of WGA Sepharose equilibrated with the same buffer. Incubation was performed at room temperature for 2 h under rotation. After sedimentation and washing of the pellet, the proteins were eluted from WGA Sepharose by three successive volumes (3 × 400 μl) of 10 g/100 ml *N*-acetyl-D-glucosamine in this buffer. The three elution fractions were pooled, supplemented with 120 μl of Complete solution, and submitted to a final ultrafiltration in a Centricon YM30 device as described above for samples not treated by WGA Sepharose. All the subsequent steps were identical.

Isoelectric focusing of the retentates

The day of the IEF run, the retentates were thawed at room temperature and, if necessary, diluted with 50 mM Tris/HCl, pH 7.4, so that an EPO level of 1500 IU/L was never exceeded. A final volume of 20 μl of the different samples was then heated at 80 °C for 3 min and supplemented with 2.2 μl of 10% Tween 80. In some experiments, instead of being heated, the samples were supplemented with 2 μl of 1.5 mM pepstatin.

The rHuEPO solutions were prepared in 1 g/100 ml bovine serum albumin (BSA) and 50 mM Tris/HCl, pH 7.4, at a final concentration of 600 IU/L. Samples of 20 μl were supplemented with 2.2 μl of Tween 80 before IEF.

IEF was performed in 1-mm-thick 5% T, 3% C polyacrylamide gels containing 7 M urea, 2% (w/v) 2–4 and 2% (w/v) 4–6 ampholytes, and 5 g/100 ml sucrose.

After prefocusing at 250 V and 8 °C for 30 min, using 2% 6–8 ampholytes as catholyte and 0.5 M H3PO4 as anolyte, the samples (20 µl) soaked onto rectangular pieces of filter paper were applied at 0.5 cm from the cathodal edge of the gel. Electrophoresis was run on the Multiphor II Electrophoresis system (Amersham–Pharmacia) at 1 W/cm of the gel length. The migration width was 9 cm. The run was stopped at 4000 Vh.

Immunoblotting

After the IEF run, the gel was submitted to semidry blotting in 25 mM Tris and 192 mM glycine at 1 mA/cm^2 of membrane for 30 min. An intermediate Durapore membrane was interposed between the blotting Immobilon-P membrane and the gel to prevent sticking. As soon as the transfer was over, the blotting membrane was incubated in 5 mM DTT PBS for 1 h at 37 °C. After a brief rinsing in PBS, the membrane was saturated in 5 g/100 ml nonfat milk PBS for 1 h at room temperature. After it had been incubated in a 1/1000 dilution of the anti-EPO antibody (primary antibody) in 1 g/100 ml nonfat milk PBS for 1 h at room temperature, the membrane was washed in six changes of 0.5 g/100 ml nonfat milk PBS. Double-blotting (DB) was then absolutely necessary to prevent nonspecific binding of the secondary antibody to the urinary proteins. This was performed as previously described [7]. Briefly, the blotting membrane was assembled with a second Immobilon-P membrane (DB membrane) and submitted to semidry transfer in 0.7% (v/v) acetic acid, at 1 mA/cm^2, for 10 min, so that the DB membrane was facing the cathode. All the subsequent steps concerned the DB membrane which was saturated in 5 g/100 ml nonfat milk PBS for 1 h at room temperature and rinsed briefly in PBS. The membrane was then incubated in a 1/4000 dilution of biotinylated anti-mouse IgG antibodies in 1 g/100 ml nonfat milk PBS at 4 °C for 15 h. After washing in six changes of 0.5 g/100 ml nonfat milk PBS, it was incubated in a 1/2000 dilution of streptavidin:biotinylated peroxidase complex in 1 g/100 ml nonfat milk PBS for 1 h at room temperature and washed in six changes of PBS.

In some experiments, classical immunoblotting was performed as described above for double-blotting except that the semidry transfer in acetic acid was omitted.

After its final washing, the membrane was covered by the chemiluminescent substrate (30 µl/cm^2), prepared as indicated by the manufacturer, and placed in the dark room of a charge-coupled device camera (Fuji). A first exposure of 3 min was tested to evaluate the obtained intensity. In most of the cases, a second exposure of 20 min was made after a transparent sheet of plastic had been layered onto the membrane. Profiles corresponding to the isoelectric patterns were obtained using "AIDA 1D-Evaluation" software from Fuji.

Results

Preliminary experiments to test the behavior of rHuEPO during ultrafiltration had been performed. Solutions of rHuEPO in 0.1 g/L BSA submitted to ultrafiltration at neutral (7.3) and acidic (4.8) pH conditions had shown that, whatever the pH, EPO was recovered in the retentate, whereas the filtrate was devoid of it. The results were quite different when rHuEPO was diluted in urine. Whereas a high recovery of the hormone in the retentate was obtained when ultrafiltration was performed at neutral pH, low to zero (depending on the urine sample) recoveries were observed under acidic conditions (data not shown).

The aspartic proteases present in urine were strongly suspected to be responsible for EPO degradation during ultrafiltration under acidic conditions. From this moment onward, 3.75 M Tris/HCl, pH 7.4, was systematically added to urine samples beforehand. This neutralized the pH of any acidic urine, with the aim being to inactivate the aspartic proteases. Because it was not possible to rule out EPO degradation by proteases active at neutral pH in some urine samples, however, a mixture of antiproteases with broad-spectrum activity (Complete solution) was systematically added to the urine samples before ultrafiltration and to the washing buffer of the retentate.

Under such conditions, EPO was finally concentrated from 200 to 1000 times in the final retentate which was then submitted to isoelectric focusing and immunoblotting of EPO. The sensitivity of the detection was tested using classical immunoblotting following IEF of pure CHO rHuEPO (Fig. 1). This showed that the recombinant hormone was composed of at least five isoforms in a p*I* range of 4.4–5.1 (in the presence of urea)

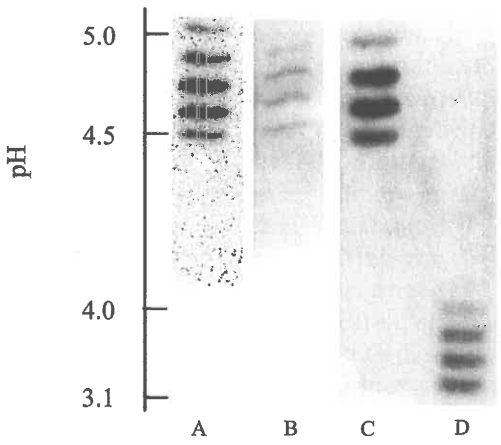

Fig. 1. IEF patterns of pure rHuEPO detected by classical immunoblotting: Epoetin β (A), Epoetin α (B, C), and Darbepoetin α (D). The same quantity of Epoetin α (10 mIU, 84 pg) was run in B and C, treatment of the blotting membrane by DTT before probing by the anti-human EPO antibody (C); no treatment (B). Anode is at the bottom of the figure.

for Epoetin α, and one additional more-basic isoform in the case of Epoetin β. The Darbepoetin, due to its two supplementary N-linked oligosaccharide chains, was much more acidic and gave rise to five bands located in a p*I* range of 3–3.9. Detection was about three times more sensitive if the blotting membrane was incubated in DTT just after the semidry transfer. Using this reducing treatment, the sensitivity achieved was about 0.2 mIU (1.7 pg) per band, which was sufficient to investigate the EPO patterns in the retentates from most of the ultrafiltered urine samples. However, when the retentates obtained from urine samples were analyzed, two kinds of problems were observed. First, as previously described, a strong nonspecific binding of the secondary antibody to some of the urinary proteins was observed after classical immunoblotting, so that the isoforms of EPO were completely masked by unrelated proteins (Fig. 2A). The double-blotting process was thus essential to prevent the urinary proteins from interfering with the detection of EPO [7]. Second, once the nonspecific signal had been eliminated, no EPO was detected following IEF of the retentates—despite sufficient levels—as ascertained by ELISA. This suggested that EPO was degraded during the IEF run. The ultrafiltration experiments had suggested that it was essential to protect EPO from aspartic proteases. Since the pH gradient of the IEF gel was 2–6, it seemed possible that urinary aspartic proteases, present in the retentates that were applied to the gel, were activated during the run and responsible for the disappearance of urinary EPO. Indeed, addition of pepstatin to the retentates just before the IEF step proved to be sufficient to protect EPO from degradation. Heat treatment of the retentates before the run, instead of pepstatin addition, was tested also. As shown in Fig. 2B, whereas "blank" lanes were obtained in the case of retentates applied directly onto the IEF gel, clear EPO profiles were observed when the same retentates were added with pepstatin or heated at 80 °C for 3 min before the run (Figs. 2C–E). All subsequent experiments were performed using the heat treatment, which proved to be unfailingly efficient in protecting EPO from degradation during the run.

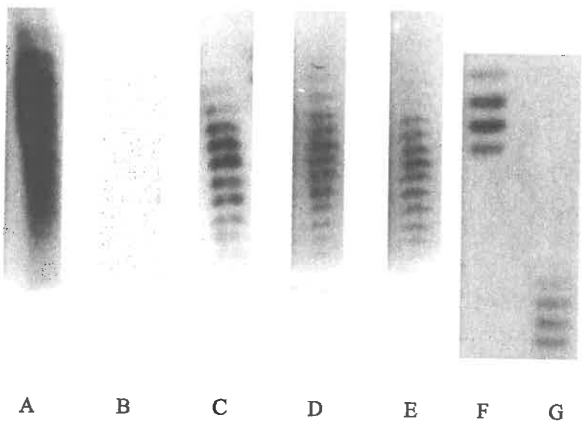

Fig. 2. IEF patterns of natural EPO obtained from urine retentates after classical immunoblotting (A), double-blotting without heat treatment of the retentate before the run (B), and double-blotting with heat treatment of the retentate before the run (C–E). For comparison, the IEF patterns of pure rHuEPO Epoetin α and Darbepoetin α are shown in F and G, respectively. Anode is at the bottom of the figure.

Under such conditions, the isoelectric patterns of natural EPO observed in urine samples from various individuals proved to be highly heterogeneous, being composed of about 10–15 isoforms in a p*I* range of 3.8–4.7 (in the presence of urea). Although some differences were noted between individuals, all natural urinary EPO patterns were clearly different from those of the various recombinant patterns. Some patterns comprised minor bands colocated with the recombinant isoforms, but in all cases, the major isoforms presented p*I*s that were more acidic and more basic than Epoetin and Darbepoetin, respectively (Figs. 2F and G). In some cases where the total protein content of the retentates was particularly high (more than 5 g/100 ml), arc-shaped bands resulted from the gel overloading. This was corrected by treating the retentates with WGA Sepharose, which considerably lowered the protein concentration in the samples applied to the IEF gel. As shown in Fig. 3, the straightness of the bands was significantly improved by this procedure. To be sure that this treatment was not selective for some of the EPO isoforms, a sample with low protein content was prepared according to the two different procedures. In both cases, the corresponding patterns were composed of straight bands that could be easily integrated and compared (Figs. 3c, c′, e, and e′). This showed that the distribution of the relative intensities of the bands was not significantly affected by the WGA Sepharose treatment.

A striking transformation in the urinary EPO pattern resulted from the administration of recombinant hormone Epoetin, reflecting the presence of the injected drug in urine. In some cases, during the first week of the rHuEPO treatment, a transitory enlarged microheterogeneity of the banding pattern (p*I* 3.8–5.1) with additional more basic isoforms (p*I* 4.4–5.1) was noted, which corresponded to the superimposed patterns of natural and recombinant EPO (Fig. 4B). After 3 weeks of treatment, however, the patterns were very similar to the pattern of the injected hormone, being mainly composed of isoforms in a p*I* range of 4.4–5.1 and, in some cases, an additional minor more acidic isoform (Fig. 4C). Such characteristic patterns were observed over the 4 days following an injection.

Discussion

Several difficulties have to be circumvented to obtain reliable images of the IEF patterns of EPO in urine.

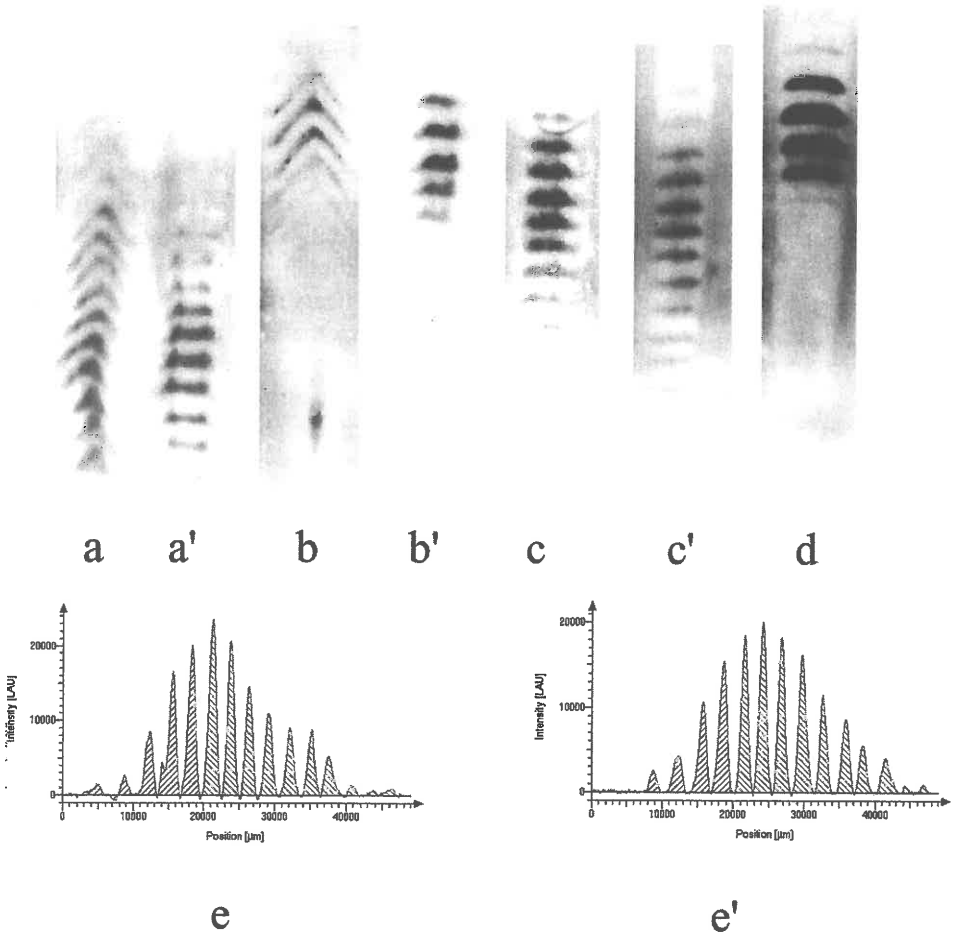

Fig. 3. IEF patterns of urinary EPO obtained from three different samples (A, B, C) prepared by ultrafiltration including (a′, b′, c′) or not including (a, b, c) the treatment by WGA Sepharose. Samples A and B showed the presence of natural and rHuEPO, respectively (see text below), and both presented high protein contents. Sample C, presented low protein content. The integrated profiles corresponding to c and c′ are shown in e and e′, respectively. For comparison, the IEF pattern of pure rHuEPO (Epoetin α) is shown in d. Anode is at the bottom of the figure.

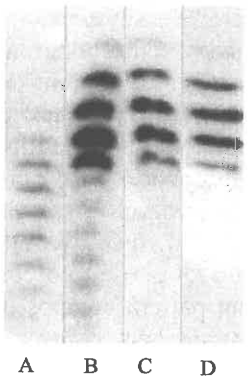

Fig. 4. IEF patterns of urinary EPO: natural EPO (A), 24 h after a first injection of Eprex (B), 24 h after a seventh injection of Eprex (2-week treatment) (C). For comparison, the IEF pattern of pure rHuEPO (Epoetin α) is shown in D. Anode is at the bottom of the figure.

The level of this hormone in urine is physiologically very low and is not increased by repeated injections of 20 IU/kg (unpublished results). Thus, urine must necessarily be concentrated. This is achieved by ultrafiltration through a membrane with a nominal MWCO of 30,000 Da. Though this is just below the molecular weight of EPO (about 34,000 Da), no passage of the hormone through the membrane was observed and thus this MWCO was selected to facilitate the elimination of smaller urinary proteins in the filtrate. Filtrate has no interest for EPO analysis but can be used for antidoping control concerning small molecules such as anabolic agents, diuretics, stimulants, or narcotics, and this may be useful in cases of small volumes of available urine.

This step has to be performed carefully; otherwise EPO may be drastically degraded due to the presence of proteases in urine. Indeed, various proteases have been described in urine: metallo proteases such as MMP-2 and MMP-9 [9], and gelatinase [10], serine proteases such as tonin [11], and aspartic proteases such as napsin A [12] and cathepsin D [13]. Since EPO degradation during ultrafiltration was observed in our experiments when acidic conditions were applied, it appears that aspartic proteases are very likely implicated. The

involvement of cathepsin D in the degradation of β_2-microglobulin in acidic urine has been reported [13], and it is possible that this protease is involved in the degradation of EPO also. Indeed, two of the specific sites cleaved by this enzyme (Tyr–Phe and Leu–Tyr) are present in the peptidic sequence of EPO and the molecular weight of the enzyme, 45,000 Da, results in its coconcentration with the hormone in the retentate during the ultrafiltration. Whatever aspartic proteases are involved in EPO degradation, they are efficiently inactivated by neutralizing the pH of urine before ultrafiltration. At the same time, an addition of anti-serine, -thiol, and -metallo proteases prevents the potential action of other types of proteases. Under such conditions, EPO is sufficiently concentrated for the subsequent IEF step.

The IEF step itself must be performed carefully because of the aspartic proteases reactivated by the acidic pH gradient. If these proteases are not neutralized before IEF, EPO is degraded during the run. This indicates that the respective p*I* of the proteases and the hormone are close enough to allow sufficient contact during the run. That pepstatin is sufficient to protect EPO from this degradation corroborates the implication of aspartic proteases. Heating the sample at 80 °C for 3 min before the run appears to be an efficient protective measure against EPO degradation by denaturing the proteases. The high thermal stability of EPO has been reported, related to its carbohydrate content [14]. We observed that its p*I* is not affected by the heat treatment, as shown by the well-preserved profile of the pure recombinant hormone after such treatment. On the other hand, this indicates that the binding of the AE7A5 antibody used for immunoblotting is not affected by the heat treatment of EPO.

The combination of an amplified (biotin:streptavidin) detection and a chemiluminescent signal provides good sensitivity that is further upgraded by incubating the blotting membrane in dithiothreitol before probing with the primary antibody. Since the AE7A5 anti-EPO antibodies used bind to an epitope within the first 26 amino acids of the molecule, it is probable that the reduction of the disulfide bridge between cysteinyl residues Cys 7 and Cys 161 makes this epitope more accessible to the antibody. Finally, a sensitivity of about 0.2 mIU (1.7 pg) per band is achieved. Assuming a mean concentration factor of 500 by ultrafiltration, the minimal concentration of EPO in urine must be about 0.4 IU/L (3.36 ng/L) to be detected.

In addition to sufficient sensitivity, the specificity of the immune detection of EPO proved to be the most difficult goal to achieve. Due to a strong nonspecific adsorption of the secondary antibodies used, it is not possible to get reliable images of the EPO isoforms that are present in urine samples. Only the double-blotting process that has been developed in these circumstances solves this problem [7].

In the case of samples with high protein contents (urine samples for antidoping control are very often taken after an intensive physical exercise that increases proteinuria), treatment by WGA Sepharose during ultrafiltration improves the straightness of the bands composing the pattern without disturbing the distribution of their relative intensities. Indeed, albumin, not being glycosylated, has no affinity for this lectin and is thus eliminated from the final retentate. This step efficiently lowers the protein content of the sample that is applied to the IEF gel, whereas EPO, which presents a very high content in GlcNAc residues, is retained with a recovery of more than 60%. The well-preserved distribution of the bands after this treatment shows that WGA Sepharose has no apparent selectivity for any of the different isoforms of EPO.

The p*I*s observed for purified Epoetin (4.4–5.1) appeared more basic than those described by Imai et al. (3–4.2) [15]. However, no urea is mentioned in the composition of the IEF gels used by these authors and this may explain the more acidic p*I* obtained. Under our conditions, the IEF gels contain 7 M urea and the p*I* observed for the recombinant CHO EPO are closer to those reported by Davis et al. (4.2–4.6) [16] in the presence of urea.

The most striking feature is the clear difference observed between the patterns obtained from untreated subjects (natural urinary EPO) and those from the different recombinant hormones. In comparison with Epoetin α and β, natural urinary hormone is mainly composed of more acidic isoforms that are missing in the recombinant patterns. This agrees with the greater electrophoretic mobility at pH 8.6, already described for natural urinary EPO in comparison with recombinant CHO hormone by Wide et al. [3]. In contrast, the isoforms of Darbepoetin α are more acidic than the natural isoforms and this can be easily explained by the presence of two additional sialylated oligosaccharidic chains which characterize this recombinant hormone. The origin of the difference between natural urinary EPO and Epoetin α or β, however, is not clear. Both hormones present the same proteic moiety but it undergoes an extensive posttranslational N-glycosylation at Asn-24, Asn-38, and Asn-83 and an O-glycosylation at Ser-126. The N-glycosylation gives rise to a complex and heterogeneous branching pattern composed of di-, tri-, and tetra-antennary glycans comprising a variable number of acetyllactosamine repeats and terminal sialic acid residues. The heterogeneity in the number of sialic acid residues is reflected in the multibanding isoelectric pattern of the hormone. The maximal possible number of sialic acid residues is 12 on the N-linked (3 tetrasialylated, tetra-antennary) oligosaccharides in both hormones [5] and 1 or 2 on the O-linked oligosaccharides in the case of urinary and recombinant EPO, respectively [17]. The tetrasialylated N-linked oligosaccharides have been shown to be the prevalent forms in recombinant

CHO EPO [18–20]. Thus, the more acidic isoforms of natural urinary EPO cannot be imputed to supplementary sialic acid residues. Deamidation may be involved in the microheterogeneity of EPO, which comprises 3 Asn residues not glycosylated and 7 Gln residues. It is well known that some nonenzymatic deamidation may occur during the storage or preparation of samples [21]. However, all the urine samples were submitted to the same analytical procedure, and the differences in the EPO patterns in urine samples treated and untreated subjects cannot be imputed to some different deamidation process occurring during analysis. Furthermore, attempts to deamidate EPO by incubation at alkaline pH at 37 °C for 24 h did not result in any change in its IEF pattern (data not shown). The presence of small amounts of oligosaccharides containing both sialic acid residues and sulfate groups has been suggested in natural EPO and rHuEPO from CHO cells [22] and sulfation of some of the GlcNAc residues of rHuEPO from baby hamster kidney cells has been recently reported [23]. Furthermore, the sulfated species may be more prevalent in natural urinary than in CHO rHuEPO [24]. This would agree with the more acidic isoforms observed in the case of urinary hormone.

The mechanism of EPO elimination is not well known. Bone marrow [25] and kidney [26] have been shown to contribute, respectively, significantly and to a small extent. Our results indicate that administered Epoetin α (or β) is excreted in urine without noticeable change in its isoelectric profile. This observation is of particular interest for antidoping applications since it allows the detection of recombinant EPO in urine [8]. This method has been thus proposed for antidoping control after having been tested in a large control population study that included different athletes to assess the influence of ethnic origin, sex, age, physical exercise, and erythropoiesis-stimulating situations (altitude, hypobaric chambers) on the natural urinary EPO pattern. The results of this study and those of administration trials using the different recombinant hormones will be published at a later date.

By enabling the investigation of the urinary IEF profiles of EPO, this method may also lead to new insights in physiology and pathology.

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Protocol

Double-blotting: a solution to the problem of nonspecific binding of secondary antibodies in immunoblotting procedures

Françoise Lasne*

Laboratoire National de Dépistage du Dopage, 143 avenue Roger Salengro, 92290 Châtenay-Malabry, France

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Abstract

"Double-blotting" (DB) has been developed to overcome the problem of nonspecific binding of secondary antibodies in immunoblotting (IB). After it has been probed by the primary antibody, the membrane with the blotted proteins is assembled with a second blank membrane and submitted to a second blotting under acidic conditions. The primary antibody molecules are thus desorbed from their corresponding antigen and transferred onto the second membrane, whereas the antigen and the interfering proteins remain bound to the first one. The second membrane can then be probed by the secondary antibodies without the risk of nonspecific binding. This method has been developed for the study of erythropoietin (EPO) in concentrated urine since a strong nonspecific binding of biotinylated secondary antibodies to some urinary proteins is observed using classical IB protocols. However, its concept makes it usable in other applications that come up against this kind of problem.

Keywords: Double-blotting; Immunoblotting; False positives; Secondary antibodies; Erythropoietin; Transthyretin

1. Type of research

Nonspecific interactions between blotted proteins and unrelated secondary antibodies generate false positives in immunoblotting (IB) techniques. Some procedures have been developed to reduce this adsorption but they may work in specific applications and be ineffective in other ones. Gershoni (1988) has emphasized the difficulty of resolving such a problem. By isolating on a new membrane (DB membrane), the primary antibody from the blotted proteins, the process eliminates the interfering proteins and makes impossible the nonspecific binding of the secondary antibody (Lasne, 2001). Thus, it may be considered as a general solution to the problem.

2. Time required

- Whole protocol: 1 h 10 min

The double-blotting (DB) process is interpolated in a classical immunoblotting procedure between the

Abbreviations: PBS, phosphate-buffered saline; EPO, erythropoietin; rHuEPO, recombinant human erythropoietin; PVDF, polyvinylidene fluoride; IB, immunoblotting; DB, double-blotting.

* Tel.: +33-1-46-60-28-69; fax: +33-1-46-60-30-17.
E-mail address: f.lasne@lndd.com (F. Lasne).

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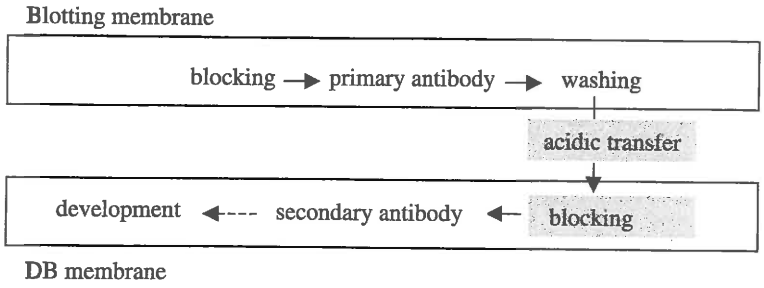

Fig. 1. Positioning of the double-blotting process into an immunoblotting procedure. The additional steps specifically related to DB are grey-coloured.

primary and the secondary antibodies probing (Fig. 1). It consists of two additional steps:

- Acidic transfer of the primary antibody onto a new membrane (DB membrane): 10 min
- Blocking of the DB membrane membrane: 1 h
- The other steps are specific of the application and are unmodified.

3. Materials

- Special equipment: semi-dry transfer apparatus
- Chemical and reagents:
  - Immobilon P (Millipore) polyvinylidene fluoride membranes (PVDF), 0.45 μm pore size
  - Durapore (Millipore) hydrophylic PVDF membranes, 0.65 μm pore size
  - filter paper
  - 0.7% (v/v) acetic acid solution
  - phosphate-buffered saline (PBS), pH 7.4

All the other reagents are those used for the specific immunoblotting application involved: blotting membrane, blocking and washing buffers, primary and secondary antibodies solutions, possibly amplification system, development reagents.

4. Detailed procedure

1. Proceed to the usual blotting, blocking, primary antibody probing and washing steps of the blotting membrane. Note that DB has been developed using PVDF as blotting membrane and has not been tested with other types of membranes.
2. Cut two stacks of filter paper, a Durapore (intermediate membrane) and an Immobilon P (DB membrane) membranes to the dimensions of the blotting membrane. Condition them in 0.7% acetic acid: just immerse the Durapore in the acidic solution for at least 10 min, prewet the Immobilon P membrane in methanol for 3 s, rinse in water for 2 min before equilibrating in acidic solution for 10 min. The stacks of filter paper are wetted in acetic acid solution by capillarity.
3. Layer the blotting membrane onto a first stack of filter paper and cover it with the intermediate and DB membranes successively. Quickly put the second stack of filter paper onto the DB membrane to prevent the membranes from drying. Position this sandwich in the semi-dry electrophoretic blotting instrument with the blotting membrane and the DB membrane facing the anode and the cathode, respectively (Fig. 2).
4. Apply a constant intensity of 0.8 mA/cm^2 for 10 min.
5. Disassemble the membranes.
6. Rinse the DB membrane quickly in two changes of PBS.
7. Proceed to the blocking of the DB membrane according to your usual procedure.
8. Note that after the acidic transfer, the blotting membrane (on which the blotted proteins are retained) can be kept in PBS if a new probing with the same or another primary antibody is planned.

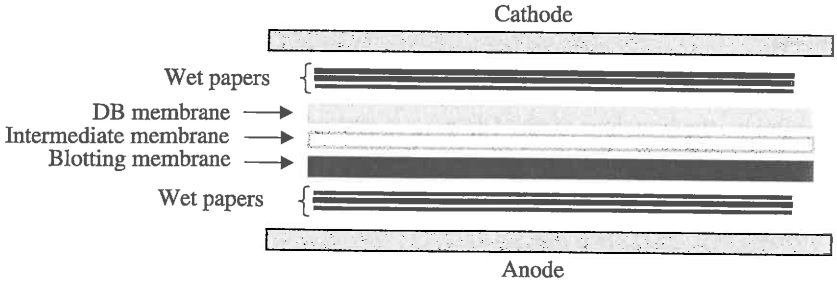

Fig. 2. Experimental set-up for DB.

9. Proceed to the usual steps of your application from the probing with the secondary antibody to the final development, on the DB membrane.

5. Results

The results are illustrated by two different applications which give rise to a nonspecific binding of the secondary antibody when classical immunoblotting is used. These are immunodetection of erythropoietin (EPO) and transthyretin following isoelectric focusing of concentrated urine and serum, respectively.

The specific reagents used in the case of EPO are:

- Primary antibodies: monoclonal mouse anti-human EPO (clone AE7A5 from R&D)
- Secondary antibodies: biotinylated goat anti-mouse IgG (H+L) (2813 CE from Valbiotech)
- Amplifying systems: streptavidin/biotinylated peroxidase complexes (GO14-61 from Biospa)
- Development system: chemiluminescence (Covalight from Covalab)

The reagents used in the case of transthyretin are:

- Primary antibodies: polyclonal rabbit anti-human transthyretin (A 002 from Dako)
- Secondary antibodies: gold-conjugated goat anti-rabbit IgG (H+L) (particle size: 20 nm) (BL GAR20 from Biocell)
- Development system: silver enhancing kit (SEKB from Biocell)

In both applications, the blocking buffer is 5% (w/v) nonfat milk in PBS buffer.

When concentrated urine samples are submitted to classical IB following IEF, a nonspecific protein pattern is observed, corresponding to the binding of the secondary antibody on some of the urinary proteins focused in this pH interval. Therefore, it is not possible to distinguish the bands corresponding to the urinary EPO (Fig. 3, A1). The same problem occurs in the case of serum transthyretin (Fig. 3, B1).

In both cases, the specific isoelectric pattern of the protein analysed is obtained by using the DB process (Fig. 3, A2 and B2).

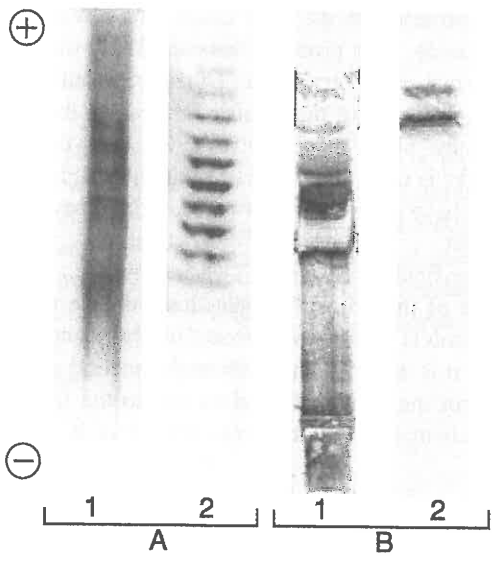

Fig. 3. Isoelectric patterns of: (A) urinary endogenous EPO; (B) serum transthyretin. Images obtained: (1) without DB; (2) with DB.

6. Discussion

In response to the problem of nonspecific binding of the secondary antibody, the principle of the DB is to transfer the primary antibody separately from the blot membrane to a new support called the DB membrane, whereas the antigen and the interfering proteins are retained on the blot membrane.

The dissociation of the primary antibody from the blotted antigen is induced by the acidic pH used for the process. Since the acidity does not affect hydrophobic interactions with PVDF, the antigen and the interfering proteins are retained on the blot membrane.

Due to the acidic pH, the antibody is positively charged and migrates towards the cathode when an electrical field is applied. The use of an electric field speeds up the transfer of the primary antibody from the blotting to the DB membrane. However, a simple contact between the membranes (passive transfer) without applying an electric field for a prolonged time (30 min) is also usable (data not shown). In both cases, the capture of the desorbed antibody by the facing membrane occurs without any diffusion.

The interposition of the Durapore membrane between the blotting and the DB membranes has been added to the procedure previously described (Lasne, 2001). Indeed, in some cases, some "holes" have been observed in the final image obtained on the DB membrane. This problem has completely disappeared after that an intermediate Durapore membrane has been used during the electric transfer of the primary antibody. Since in our applications, only the electric transfer is used, the effects of this intermediate membrane have not been investigated in the case of passive transfer.

The final result on the DB membrane is a specific image of the probed antigen. It should be noted that the result is actually an "image" of the probed antigen since it is only the antibody on the second membrane and not the antigen that gives rise to the final signal but this image is quite representative of it.

If wanted, a second DB can be performed from the blotting membrane if this one is kept in PBS buffer after the first DB. In this case, this membrane is probed again with a primary antibody (same or different as the first time) before to be submitted to the second DB.

This procedure has been developed to study EPO isoelectric patterns in urine concentrated by ultrafiltration and has made possible the differentiation of endogenous and exogenous recombinant hormone for anti-doping control purposes (Lasne and de Ceaurriz, 2000; Lasne et al., 2002).

The DB is expected to be especially useful for investigating proteins that are present in minute amounts in complex biological media.

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Erythropoietins and Erythropoiesis
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Abuse of recombinant erythropoietins by athletes

Don H. Catlin[1], Caroline K. Hatton[1], and Françoise Lasne[2]

[1] *UCLA Olympic Analytical Laboratory; Department of Molecular and Medical Pharmacology, University of California, 2122 Granville Ave, Los Angeles, CA 90025, USA*
[2] *Laboratoire National de Dépistage du Dopage, 143, Avenue Roger Salengro, 92290 Châtenay-Malabry, France*

History

History of doping

Athletes have always sought ways to enhance their performance. In ancient times, medicinal plants were used for doping, i.e., the artificial enhancement of athletic performance. According to Philostratus, a Greek physiologist in the third century AD, "[athletes should be]...freed from the use of clay and mud and irksome medicine" [1]. He described Olympians who enhanced their athletic abilities by eating bread soaked in opium.

Some of the earliest pharmaceuticals, amphetamine, strychnine, and ephedrine, rapidly became interesting to athletes in search of increased speed in athletic events. Canal swimmers in Amsterdam were reported to have used strychnine to win races. The first documented doping fatality at the Olympics shocked the 1960 Games [2] and it involved amphetamine abuse.

"There can be no doubt that stimulants are today widely used by athletes participating in competitions; the record-breaking craze and the desire to satisfy an exacting public play a more and more prominent role, and take higher rank than the health of the competitors itself."[3] Written in 1939 by a physiologist, this quote is still apropos today and it shows the length of time athletes have had the "win-at-all-cost" mentality.

Currently, sport is coping with both drugs developed with recombinant-DNA technology and genetic manipulation. In the 1970s, when the United States Congressional hearings documented significant abuse of stimulants and anabolic steroids [4], medical organizations took anti-doping stands and widespread amphetamine abuse was described in American professional football.

Characteristics of doping

Not all athletes dope – fortunately most do not – but those who do have a profound influence on sport. Ben Johnson set a world record in the 100-meter sprint at the 1988 Games of Seoul, but he was doped with stanozolol, an anabolic steroid. Until this Games-stopping event, the hope had been that doping was confined to strength sports. The Johnson affair led to vociferous cries from clean athletes for more testing and stricter penalties, and spurred sport to devote more resources to solving the problem. In retrospect, the resources were inadequate but served to heighten awareness and to begin to solve the problem.

An axiom of drugs and sport is that some athletes will try any new drug that has the potential to enhance performance. The trials are conducted secretly and are, of course, never published or presented. Sometimes information may be shared with close partners, but generally not. Often members of the athletes' entourage are deeply involved in doping.

Doping has occurred in nearly all sports, but certain ones have predilections [5, 6]. Endurance events, such as long-distance running, cycling, swimming, and skiing events, often have participants who have tried various types of blood doping including the use of erythropoietic proteins. Short-distance speed events are known for abuse of stimulants; and weightlifting, throwing events, and several other sports have been plagued with anabolic androgenic steroid doping. Participants in shooting events used beta-blockers to improve shooting scores [7].

Development of testing

By the mid-1960s, international sport officials were very concerned about drugs and were actively exploring several approaches to deal with the problem. Until the mid-1960s, sport had used education as its main approach, but elite athletes were now beginning to receive substantial financial rewards for success and education alone was not effective. In 1967, Prince de Merode presented a survey of the problem to the International Olympic Committee (IOC). Soon thereafter, he became chairman of the IOC Medical Commission and plans were made to implement urine testing to detect doping [8]. After experimenting with urine testing at the Winter Olympics of Grenoble in 1968, the IOC implemented widespread testing at the Summer Olympic Games of Munich in 1972. In 1984, only five IOC-accredited laboratories existed; now 25 laboratories operate in 23 countries. In the early years of the program, testing was confined to the Olympic Games. It has been adopted by other competitions, and now athletes may be tested at any time during the year. Out-of-competition testing was implemented when it became clear that the ergogenic effects of drugs, such as anabolic steroids and erythropoietic proteins, persisted for many days after administration was discontinued. Without random, out-of-competition testing, athletes could discontinue doping before announced in-

competition tests, thus evading detection. Approximately 10,000 samples are tested per year, and of these, approximately 50% are collected as out-of-competition tests.

The Court of Arbitration for Sport (CAS)

The Court of Arbitration for Sport (CAS) was created by the IOC in 1983 to resolve sport-specific disputes [9]. CAS provides a forum for judicial resolution of disputes among athletes, national and international sport federations, national Olympic Committees, games organizers, sponsors, and others. Disputes are presided over by a panel of three arbitrators selected from a pool of CAS arbitrators. All CAS decisions are final with nearly no possibility of appeal.

Doping cases are subject to the strict liability rule that states that: "doping is... the presence in the athlete's body of a Prohibited Substance..." [10].

Thus, if a prohibited substance is found in an athlete's body, a doping violation has occurred irrespective of any other factor [9]. At times this rule results in seemingly harsh consequences, as was demonstrated by the case of the Romanian gymnast who placed first in the Women's Individual All-Round Event at the Olympic Games of Sydney. The athlete tested positive for pseudoephedrine that was allegedly given to her by the team doctor.

The two most common subjects that are argued in CAS cases are the validity of the analytical methods used to establish that a prohibited substance has been detected in the athlete's body fluid and the chain-of-custody of the sample. The principles of both the analytical methods that may be applied and the chain-of-custody are specified in the Medical Code of the Olympic Movement [10]. During the adjudication procedure, the laboratory director typically defends the analytical results and the sample-collection officials defend the chain-of-custody issues.

The World Anti-Doping Agency

Faced with many positive test results, mounting numbers of drugs and methods to consider banning, laboratories to accredit and re-accredit, and complex issues involving new fields of science, the IOC fostered the development of a new agency, the World Anti-Doping Agency to supervise the drug-control effort. This move coincided with revelations in 1998 that many cyclists competing in the Tour de France were doped with recombinant human erythropoietin (rHuEPO). Since the IOC had been criticized for being slow to develop adequate anti-doping programs, the anti-doping agency was structured to operate outside the direct control of the IOC. It is expected that this agency, with its substantial resources, will have a major impact on the doping problem.

Doping by expanding the red cell mass

"Induced erythrocythemia" is a term used to describe expansion of the blood volume by transfusion of whole blood, transfusion of packed red cells, or by administration of erythropoietic proteins. The term "blood doping" has been used to describe any of these three methods, although obvious differences exist in the techniques and the infrastructure required to use them.

Autologous and homologous blood doping in sport

Athletes have used both homologous and autologous blood products. The typical technique for autologous transfusion involves phlebotomy, freezing the red blood cells under glycerol, allowing eight to 12 weeks for hemoglobin recovery while continuing training, and infusion of the red blood cells one to two days before the scheduled athletic event. The use of homologous blood products avoids the regeneration period, but is associated with multiple risks and adverse effects. The terms "blood packing" and "blood boosting" usually refer to doping with homologous and autologous blood products, respectively.

Rumors and anecdotes suggest that an airplane equipped with refrigeration devices was used to support doping at the 1976 Games in Montreal. The first documented case of blood doping occurred in the 1980 Olympics in Moscow when a Finnish distance runner freely admitted having received two units of blood shortly before he won medals in the 5- and 10-km races [11]. At the 1984 Games in Los Angeles, seven cyclists from the United States team were found to have blood doped [12]. One cyclist doped by autologous transfusion and had his personal-best time at the Olympic trials a month before the Games. By the time the news reached his team-mates, it was too late to use the autologous infusion paradigm, so with the aid of a physician, whole blood from relatives and unrelated donors was transfused to the cyclists in a motel room [13]. Four of the seven cyclists won medals. During the ensuing investigation, the athletes justified the act by pointing out that the IOC list of prohibited substances did not explicitly ban blood doping. Shortly thereafter, the IOC added doping with blood products to the list. The only other documented case is that of a United States skier who admitted to blood doping with autologous blood in 1987 [14].

Doping with rHuEPO in sport

Given that blood doping with transfusions is a complicated matter that requires substantial infrastructure, such as blood harvesting equipment, processing devices, and refrigeration, it is understandable that the availability of a drug, even one that required intravenous or subcutaneous injection, would be used. It is not known when doping with rHuEPO began. The secrecy and privacy that surrounds the topic precludes detailed knowledge, but it is understood that ath-

letes and their medical entourage follow pharmaceutical developments closely. Thus, it is not surprising that rumors of rHuEPO's potential for abuse by athletes first surfaced before rHuEPO was approved for marketing (1987), and that rumors of actual use circulated before the 1988 Winter Games of Calgary (Tab. 1).

In the late 1980s, a cluster of up to 20 deaths occurred among elite cyclists in the Netherlands and Belgium. The story was widely reported, followed by an official investigation, but no details emerged. It is speculated that rHuEPO may have been involved in these deaths [15, 16]. We were unsuccessful in obtaining the details through contacts in the sport community. Because the deaths occurred after rHuEPO received marketing approval, it was widely speculated that they were due to excessive doses of the drug. In 1990, "erythropoietin" and analogues were added to the IOC list of prohibited substances.

Although it was widely accepted that rHuEPO was being abused throughout the 1990s, it was not until the 1998 Tour de France that its use was documented. The discovery of rHuEPO and other drugs in the trunk of an automobile just before the race led to a widespread investigation that eventually resulted in recovery of many drug products, confessions from several athletes, and evidence of widespread involvement of cycling teams and coaches [17–19]. This exposé differed from other sensational drugs in sport stories (such as Ben Johnson, the track and field athlete, in 1988) in that most of the competitors were involved, not an individual athlete. This finding convinced cycling officials that rHuEPO doping was pervasive and that users and winners were highly correlated. Further, officials in other endurance sports understood that their sports were at risk. Some of the steps taken collectively by sport included further implementation of blood screening methods (discussed later in this chapter), monitoring athletes' red blood cell parameters, increased funding for research on detection, formation of expert committees, and more emphasis on the role of police authorities. A most unfortunate aspect of such developments is that success is equated with doping, thereby raising suspicions about any athlete who performs well. This belief has led drug-free athletes to speak out against doping and to pressure authorities for more effective action.

Prevalence of doping with erythropoietic proteins

It is nearly impossible to estimate the prevalence of blood doping of any kind. Only one survey has attempted to do so and commingled data on transfusion doping with rHuEPO doping. Scarpino et al. [20] interviewed 1,015 Italian athletes and reported that 7% regularly used red blood cell infusions or rHuEPO techniques and that 25% were "occasional" users. In the same study, coaches, managers, and team physicians also estimated that 7% of athletes were regular users of doping. Sport hot-lines are another source of information. Throughout the 1990s, the United States Olympic Committee operated an

Table 1. Blood doping in sport: chronology of seminal events

Year	Description of event	Venue	Place	Source
1968	Rumors of whole-blood doping	Olympic Games	Mexico City	Second-hand accounts
1976	Rumors of transfusions	Olympic Games	Montreal	Second-hand accounts
1980	5-km medallist admits blood doping	Olympic Games	Moscow	Primary account, newspaper
1980	Autologous transfusion increases performance	Key finding		Scientific literature
1984	Homologous and autologous transfusions (n = 7)	Olympic Games	Los Angeles	Public admissions
1987	Epoetin alfa approved by US FDA			
1988	Rumors of rHuEPO misuse	Olympic Games	Calgary	Newspaper
1989	Several unexplained cyclist deaths		Netherlands	Newspaper
1998	rHuEPO confiscated by authorities	Tour de France	France	Court proceedings, newspaper
2000	Practical test method for rHuEPO published	Key finding		Scientific literature
2000	rHuEPO test deployed	Olympic Games	Sydney	Official IOC reports
2002	IEF tests positive for rHuEPO	Cycling race		Court decision, CAS
2002	Darbepoetin alfa found in urine (n = 3)	Olympic Games	Salt Lake City	Official IOC reports

CAS, Court of Arbitration for Sport; IEF, isoelectric focusing; IOC, International Olympic Committee; rHuEPO, recombinant human erythropoietin.

anonymous hot-line for athletes to call to request information about drugs and sport. Committee reports indicate that the number of calls regarding rHuEPO increased during the 1990s.

The lay sport literature reports widespread doping with erythropoietic proteins in endurance sports such as track, cycling, and cross-country skiing. These reports are largely confined to elite athletes competing in major events. From the frequency of these sensational reports, one gains the impression that such doping is common, but prevalence cannot be estimated from such data. To date, neither college sport administrators nor the media have reported doping with erythropoietic proteins in the college sport population.

Now that a urine test for erythropoietic proteins (discussed later) is beginning to be deployed, some additional data will be forthcoming from sports organizations. The IOC, World Anti-Doping Agency, and international sport federations are likely to periodically make summary data available. The nature of these reports will vary, but in some cases, they will include the names of athletes and the specific events. Since late in 2000, the media have reported several cases of doping with erythropoietic proteins [21]. The media reports occur many months before the adjudication proceedings that could find that doping did not occur. By late 2002, CAS had heard two cases of alleged doping with rHuEPO. In one case, the Court upheld the laboratory result and determined that the athlete had doped with rHuEPO [22]. In the second case, CAS concluded that rHuEPO doping had not been proven due to a flaw in the analysis [23]. Although no one knows to what extent athletes use erythropoietic proteins, it is increasingly obvious from all sources of data that they are being used. Although the use currently appears to be confined to the highest echelons of endurance sport, if the history of doping with other agents (notably steroids) is any indication, their use will spread.

Does expanding the red cell mass enhance performance?

The underlying theory of doping is that increased oxygen-carrying capacity increases performance of muscle tissue by increasing oxygen supply. The ability to perform sustained aerobic exercise depends on both oxygen delivery to muscles and ability of the tissues to use it. Which of the two limits exercise capacity has been the subject of many investigations and debates [13, 24–26]. $VO_{2\,max}$, a widely accepted index of physical fitness [27], correlates with red cell mass [28], thereby supporting the hypothesis that an expanded red cell mass enhances performance by increasing the amount of oxygen delivered to muscle. The increase in blood viscosity due to increased concentrations of hemoglobin does not limit delivery, as long as the hemoglobin concentration is <20 g/L [29]. Other possible theories advanced to explain the increase exercise capacity are that the expanded blood volume leads to increased cardiac output, improved buffering capacity for the lactic acid accumulated during exercise, and enhanced heat dissipation.

A paradox exists in exercise physiology. Athlete fitness is negatively correlated with hematocrit, yet increasing the hematocrit by transfusion or by doping with erythropoietic proteins improves $VO_{2\,max}$ and performance [30, 31]. Exercise training expands plasma volume and decreases hematocrit and hemoglobin concentrations, thus causing a negative correlation between hematocrit and fitness [30–33]. Values for hematocrit, hemoglobin, and red blood cell count are in the lower range of normal for athletes [30, 34]. In one study, athletes with the lowest hematocrits had the highest aerobic working capacity and isometric adductor strength [31].

At first it was thought that the hemoconcentration that accompanies endurance competitions would lead to increased blood viscosity and that this would severely limit cardiac output and thereby limit the usefulness of rHuEPO. Hematocrit is highly correlated with blood viscosity [31]; however, because hematocrits of 50% and more are often recorded among competing athletes, other factors must be operating. In one study of athletes competing in an ultra-marathon, the immediate post-race hematocrits were not different from the baseline values, and on the day after the competition, the values were lower than baseline [30]. Apparently replenishing lost fluid and electrolytes during very long events is sufficient to avoid serious increases in viscosity.

The notion that dehydration during long endurance events will lead to hemoconcentration and increased blood viscosity and, therefore, result in athletes experiencing strokes and other complications of increased blood viscosity is difficult to confirm. No media reports exist of athletes experiencing such catastrophes during endurance events. The cluster of deaths in cyclists in the late 1980s [15] could be related to increased blood viscosity, but the athletes were not competing at the time of death.

Autologous blood infusion increases performance

Many clinical experiments have shown that transfusions increase human performance [24, 25, 35–37]. The most convincing study [25] used a double-blind, sham-infusion controlled, cross-over design. Highly trained elite athletes received 900 mL of autologous red blood cells collected and frozen approximately seven weeks earlier; 24 hours after the transfusions, the athletes experienced a 35% increase in run-time to exhaustion, a 5% increase in $VO_{2\,max}$, and a 7% increase in hemoglobin concentration. The authors concluded that the limit to aerobic activity was the transport of oxygen to muscle. In another study, this time using 10-km race-time as the outcome rather than physiological measurements, six highly trained but not elite athletes improved their mean run-times from 33.3 to 32.1 min and their hematocrits increased 5% shortly after receiving a 400 mL autologous infusion of red cells collected 11 weeks earlier [37]. An interesting aspect of autologous infusions is that the magnitude of the improvement is related to the baseline level of fitness. Individuals who are moderately fit experience the greatest improvement while

individuals who are poorly fit or extremely fit experience less improvement in maximal oxygen uptake [36]. Improvement in human performance is a consistent feature of blood transfusions, provided that the amount of red cells infused is sufficient to increase serum hemoglobin concentration and hematocrit and thereby deliver more oxygen to working muscles. The improvements can be shown in race-times, fixed-run times, $VO_{2\ max}$, run-times to exhaustion, and other measures of performance. Accordingly, sport officials search for ways to deter those athletes who are determined to dope with transfusions or any other means to increase the delivery of oxygen to muscles.

rHuEPO enhances performance in healthy subjects

It is well known that rHuEPO administered to patients with anemia significantly improves fatigue, physical symptoms, and physical performance [38]. (See Chapters 9 and 10 for further information.) In the present context, the question is whether or not rHuEPO enhances physical performance in healthy subjects and, particularly, in athletes. Since the release of rHuEPO almost 15 years ago, few studies on have been done with athletes and most of the studies that have been done have involved its effect on red cell indices and other markers.

Two placebo-controlled studies have shown that rHuEPO increased $VO_{2\ max}$ by 6.0% to 7.7% after three to four weeks of subcutaneously administered rHuEPO (150 U/kg/week) [39, 40]. Typically, the weekly dose is administered subcutaneously in doses of 150 U/kg/week. At a higher dose (180 to 210 U/kg/week, administered subcutaneously), the $VO_{2\ max}$ increase was similar (7%) [41]. These studies, which used recreational athletes, establish that doses of rHuEPO that are sufficient to increase the hematocrit to nearly 50% also increase the $VO_{2\ max}$ up to 7%. Anecdotal reports, however, indicate that after a few weeks of modest doses (150 U/kg/week), athletes lower the weekly dose to approximately 60 U/kg/week. To determine the effect of such a regimen, after three weeks at 150 U/kg/week, investigators [40] decreased the dose to 60 U/kg/week for an additional five weeks and found that the improvement in $VO_{2\ max}$ (4.7% to 9.7%) and increased hematocrits continued. This study and a similar low-dose study [42] provide support for the anecdotal reports from athletes, and confirm that maintenance doses of 60 U/kg/week are capable of enhancing aerobic performance for at least three weeks after discontinuation of drug. The question of how long the benefits last after rHuEPO is discontinued will be discussed.

Adverse effects of rHuEPO in athletes

Unlike studies in patients with anemia, no surveys have been done of the adverse effects of rHuEPO in athletes. Athletes tend to be healthy and that may

offer some protection against the known side-effects in patients with anemia; however, athletes are deliberately increasing their hematocrits to levels associated with thromboembolic and other complications. Despite the increases, only one case has been reported that appears to link rHuEPO to a cerebral thrombosis. The athlete admitted to using rHuEPO and other drugs [43]. Athletes who use rHuEPO are at risk of developing true iron deficiency, functional iron deficiency, or iron overload. Of course, careful professional medical management could avoid these complications. Cazzola reports that an investigation by Italian magistrates [44] reveals that some professional cyclists have evidence of iron overload with ferritin levels in excess of 1,000 ng/mL [45]. The investigation also provided data consistent with a risk of post-treatment blunted production of endogenous EPO [45]. In addition Berglund and Ekblom [42] have studied the effect of rHuEPO on the blood pressure of athletes. The systolic and diastolic blood pressure values at rest were unchanged after rHuEPO treatment; however, systolic blood pressure markedly increased during submaximal exercise. The initial and final values were 177 mmHg and 191 mmHg, respectively [42]. The propensity of athletes to titrate their hematocrits to high levels and to take rHuEPO without adequate medical supervision, together with their risk for iron disorders and exercise-induced increased systolic pressure, make it likely that more adverse effects are occurring than are reported in the medical literature. This under-reporting is inherent to the secretive nature of doping.

Detecting erythropoietic proteins in body fluids

Direct and indirect tests

Sport classifies tests for doping substances as direct or indirect. A direct test identifies the substance by an unambiguous method such as gas chromatography-mass spectrometry, whereas indirect tests measure, for example, the serum concentration of markers that correlate with the use of a prohibited substance, without directly identifying the substance. Direct tests sufficed for many years, but now that doping includes endogenous steroids and glycoproteins, a variety of new strategies and indirect tests have been developed [46]. Indirect tests have not been used to declare that an athlete has used a substance, but it is hoped that with sufficient validation, these tests could become definitive.

Detecting the use of pharmaceutical testosterone was a challenge with gas chromatography-mass spectrometry because pharmaceutical and endogenous testosterone could not be distinguished. The problem was partly solved by performing longitudinal tests of the urinary steroid profile and by determining the $^{13}C/^{12}C$ ratio of urinary testosterone by isotope ratio mass spectrometry [47]. The glycoproteins present a special challenge because, to date, it has not been practical to develop a mass spectrometry-based method that is sensitive

enough to detect either rHuEPO or recombinant human growth hormone (rHuGH) in human urine. Sonksen and colleagues attempted find a group of blood parameters that would indicate recent use of rHuGH. After collecting baseline blood samples, they administered rHuGH for several days and monitored serum concentrations of six substances known to be influenced by rHuGH [48] A composite score indicative of recent use of rHuGH was developed. The results showed good separation between the scores of the placebo-treated and rHuGH-treated subjects. The investigators intend to expand the studies to a much larger number of subjects and to determine the variability of the markers in various ethnic groups. The hope is to find a composite score that is so convincing that the indirect test is considered definitive. Until 2000, no practical direct test existed for rHuEPO; therefore, certain sports implemented indirect tests to identify potential users of rHuEPO.

Indirect tests for doping with erythropoietic proteins

The hematocrit 'health' test

The International Cycling Union, concerned that EPO had pervaded elite competition, declared that athletes could not compete with a hematocrit >50% and >47%, for men and women, respectively [49, 50]. The International Ski Federation implemented a similar rule based on hemoglobin values exceeding 185 g/L (men) or 165 g/L (women). One strategy was to determine the values immediately before an event and withhold the athletes from competition if the limits were exceeded. The hematocrit cut-off values have been changing, but generally they are 50% for men and 47% for women. The hematocrit test has been called a "health test" because it is considered dangerous for an athlete to compete if the hematocrit is greater than the cut-off. The term "health test" pre-empts legal action because the athlete is not declared a drug user, he/she is only unable to compete. The ban on competition is lifted after 15 days, provided that the hematocrit has decreased to acceptable values.

Aside from the health issue, the argument in favor of a hematocrit test is that by imposing an upper limit, the test prevents excessive use of rHuEPO. An argument against the hematocrit test is that it would discriminate against individuals who have naturally high hematocrit values [51, 52]. Partially countering this argument is the finding that of 334 hematocrits determined on 34 professional cyclists before rHuEPO was available, the values ranged from 39% to 48% (mean: 43%) [53]. In addition, a protocol was developed to determine if an athlete's hematocrit naturally exceeds 50%. Postural changes in hematocrit are avoided by taking samples after sitting for 15 min. Increased hematocrits due to dehydration have not been a significant problem [30]. Despite the controversial nature of the test, its use is having the intended effect: Hemoglobin values among elite cross-country skiers increased dramatically from 1994 to 1996 and declined after the test was implemented [54].

A disadvantage of the hematocrit test is that it draws attention to the benefits of an increased hematocrit, thus tempting athletes with a natural baseline <50% to find a way to increase it. Indeed, it is common lore that some athletes check their hematocrits using portable centrifuges and self-administer saline infusions and phlebotomy if the values are too high. They are also reported to take anticoagulants to prevent thromboembolic events.

Hypochromic macrocytes

Casoni et al. [55] studied red cell indices after administering rHuEPO every other day at an average dose of 15 U/kg/day for up to 45 days. The parameters that changed the most were mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH). The authors defined red cells with MCV > 128 fL and MCH <28 pg as hypochromic macrocytes and proposed a cut-off of 0.6% to distinguish rHuEPO users from healthy controls. No further work on this particular index has been published.

Serum transferrin receptor (TfR) and Ferritin

Serum levels of serum transferrin receptor (TfR) and ferritin are regulated by cellular iron status, and cellular iron uptake is facilitated by TfR-mediated endocytosis. As a result of externalization of TfR during the endocytic cycle, a soluble form of TfR can be detected in serum. Thus, the major determinants of serum TfR concentration are cellular iron demands and red blood cell proliferation rate. Since rHuEPO expands the red cell mass, it was logical to determine if serum TfR and ferritin could serve as indirect markers of rHuEPO administration.

Several studies have shown that amounts of serum TfR increase, amounts of ferritin decrease, and that the ratio of serum TfR/ferritin increases when rHuEPO is administered [41, 56, 57]. Furthermore, these changes are detectable in most subjects for up to one week after discontinuing rHuEPO [41]. The change in serum TfR/ferritin ratio was less dramatic in subjects treated with supplemental iron [41] than in subjects who did not receive iron [56]. Thus, the absence of supplemental iron may exaggerate the sTfR/ferritin ratio. Most users of rHuEPO are likely to take supplemental iron. The specificity of the changes in serum TfR, ferritin, and sTfR/ferritin has not been evaluated in a large group of healthy normal controls of different ethnicities, subjects with disease, or under various conditions of iron supplementation, altitude, or training. Nevertheless, these markers are relatively simple to measure and they may be useful as indirect markers of the use of erythropoietic proteins, either alone or in combination with other markers.

Multiple markers of erythropoietic activity: the Australian studies

In the year before the Sydney Olympic Games of 2000, Australian scientists did an extensive series of studies designed to find a combination of blood markers that would indicate which athletes were using rHuEPO [39, 58]. The approach was to administer rHuEPO for three weeks, collect blood for analy-

ses before, during, and for two weeks after administration was discontinued, and measure a variety of potential markers in serum and blood. The data were analyzed by various statistical models that provided the optimum combination of variables and the weight to attach to each. The main study compared placebo-treated with rHuEPO-treated subjects who received 50 U/kg three times per week for 25 days [58]. The treatment group comprised 49 Australian subjects and 24 Chinese subjects.

The study found that five markers provided the most discrimination between the placebo-treated and rHuEPO-treated groups. These were hematocrit, reticulocyte hematocrit (MCV of reticulocytes multiplied by the number of reticulocytes), percent macrocytes, serum concentration of EPO, and serum concentration of serum TfR. The total score was referred to as the "on-score" [58]. By the end of the 3-week rHuEPO administration period, the "on-scores" of the placebo-treated and rHuEPO-treated groups differed significantly. Serum EPO and sTfR were particularly increased in the rHuEPO-treated group compared with the placebo group. No differences were noted between the Australian and the Chinese subjects or between men and women.

The Australian investigators also calculated the "on-score" on approximately 1,200 elite athletes from 12 countries [58]. Based on these data, they calculated cut-offs for the "on-score" values beyond which the risk of a false-positive was very low. At a meeting of experts three months before the Sydney Games [59], the "on-score" was not approved as a standalone index of use of erythropoietic proteins largely due to legal and medical concerns. It was approved, however, as a technique to indicate which urine samples should be tested by the recently developed, definitive urine test (discussed later). The rules stated that an athlete could not be declared "positive" for rHuEPO unless the "on-score" was greater than the cut-off score and the urine test showed rHuEPO. Samples could be declared "suspicious" if the "off-score" was increased or if only one of the blood or urine tests was positive. No athletes were declared positive during the Games of Sydney, but seven were reported as "suspicious" [60].

Index of recent use of erythropoietic proteins

The Australian researchers found that after rHuEPO was discontinued, the reticulocyte count and serum EPO concentrations were depressed while the hematocrit remained increased [58]. From these three parameters they calculated an "off-score" and proposed that an increased "off-score" be used to identify athletes who had recently discontinued rHuEPO. The "off-score" was increased from day three to day 13 post-administration of rHuEPO. Although the statistical certainty that the "off-score" indicated recent use was quite high, the IOC expert committee did not accept the test largely because of the legal difficulty of proving that an athlete used rHuEPO on the basis of an indirect test. The "off-score" is used today to indicate which athletes should be followed closely with other testing.

Urine EPO concentrations by immunoassay

Attempts to detect abuse of erythropoietic proteins by urine immunoassay have not been successful. Since rHuEPO and endogenous urinary EPO cannot be distinguished by the available antibodies [61], a diagnostic test would depend on urinary EPO concentrations that are far above the normal range. In addition, <5% of the dose is excreted in urine [62] and urinary EPO concentrations are affected by pH, specific gravity, and exercise [63, 64]. Small increases in urinary EPO were detected by a immunoradiometric assay after large doses (200 U/kg every other day for 10 days) [61].

Direct tests for doping with erythropoietic proteins

The isoelectric focusing test for urinary rHuEPO

A method for detecting rHuEPO in urine by electrophoresis was first described in 1995 [65]. Although this test had practical limitations, it demonstrated conclusively that the isoform pattern of urinary endogenous EPO differs from the pattern of urinary rHuEPO, and it was the first successful attempt to develop a direct test for urinary rHuEPO. Similarly in 2002, Skibeli et al. [66] isolated EPO from human serum and showed, using gel electrophoresis, that endogenous and recombinant EPO differed.

A significant improvement in practical detection occurred in when Lasne and de Ceaurriz [67] described a method for detecting rHuEPO in urine based on isoelectric focusing with immunoblotting plus one novel and critical step: a second blot ("double-blotting") [68]. After the isoforms of rHuEPO are separated by isoelectric focusing, the first blot is performed, then the membrane containing the transferred proteins is incubated with anti-EPO antibody. The second blot transfers only the anti-EPO antibodies to a second membrane, and the second membrane is incubated with a second antibody directed against the first antibody. This step markedly reduces non-specific binding and yields clear isoform patterns. After the second antibody is incubated with streptavidin-horseradish peroxidase and substrate, the emitted chemiluminescence is captured to produce an image of the gel [69].

This method shows that endogenous EPO is highly heterogeneous and is composed of a number of isoforms. Most of these isoforms are not present in the recombinant hormones. After administration of rHuEPO or darbepoetin alfa (a new erythropoeisis-stimulating protein with a longer half-life than rHuEPO), a striking change in urinary EPO pattern is observed with the appearance of new isoforms corresponding to the excretion of the injected drugs and, after sufficient doses, the disappearance of the endogenous isoforms. By analyzing the isoelectric pattern it is possible to determine if the excreted EPO is of natural or recombinant origin. Figure 1 is an electropherogram showing the band pattern of darbepoetin alfa in a urine obtained from a patient who was treated with darbepoetin alfa.

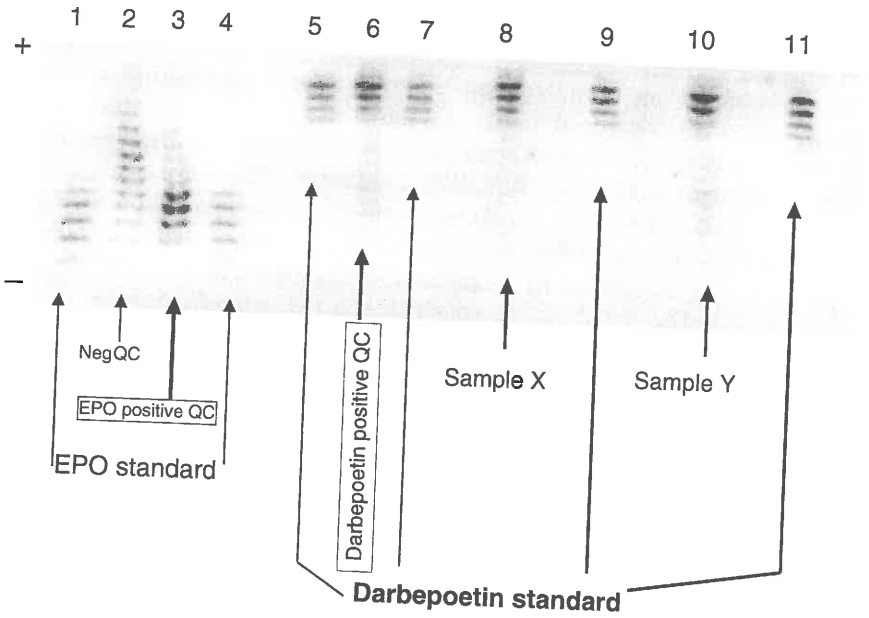

Figure 1. Electropherogram of rHuEPO and darbepoetin alfa standards; of extracts of urine obtained from drug-free control volunteers and patients treated with rHuEPO or darbepoetin alfa; and of extracts of urine from unknown individuals. The anode and cathode sides of the electropherogram are labeled + and –, respectively. The rHuEPO standard is in lane one and four and the darbepoetin standard is in lanes five, seven, nine, and 11. The negative quality control sample (lane two) is an extract of urine from drug-free volunteers showing the normal pattern of endogenous EPO. The EPO positive quality control sample (lane three) and darbepoetin positive quality control (lane six) are urine extracts obtained after the administration of rHuEPO or darbepoetin alfa, respectively. Samples X and Y are urine extracts from two unknown individuals (lanes eight and 10). The bands of endogenous urinary EPO are faint or absent in lanes six, eight, and 10.

Despite the fact that isoelectric focusing is technically demanding and may take up to three days to complete, it has generated a great deal of interest because it is a direct test that can yield conclusive results. Further, the results of the test and the underlying method have been accepted by CAS [22]. Presently, at least six IOC-accredited laboratories have implemented the test and several more are working on the method. Investigators are working on means to improve the test, to expand its use to all 25 IOC-accredited laboratories, and to further define criteria for determining if rHuEPO is present. In addition, investigators are working on ways to detect erythropoietic proteins with mass spectrometry.

Darbepoetin alfa detected at Winter Olympic Games of Salt Lake, 2002

As the IOC-accredited laboratory designated to perform the testing at the Games of Salt Lake, our laboratory began implementing the isoelectric-focusing technique for detecting erythropoietic proteins about eight months before

the Games. Since darbepoetin alfa was likely to be approved for marketing before the Games, we were aware that athletes might have access to it at the time of the Games. Accordingly, we obtained a urine sample from a patient being treated with darbepoetin alfa. As shown in Figure 1, the electropherogram of this urine sample confirmed [70] that the isoforms of darbepoetin alfa were more acidic than those of rHuEPO [71] because the former migrated to the more acidic region of the gel. This sample was important because IOC laboratory regulations require that analyses include a reference standard, a known positive quality control urine sample, and a known negative quality control urine sample. The details of the method and the main finding have been described [69, 70].

During the Games of Salt Lake, our laboratory analyzed 598 urine samples for the drugs on the IOC List of Prohibited Substances. In addition, 1,222 blood samples were obtained from athletes competing in the endurance sports of cross-country skiing, biathlon, Nordic combined, long-track speed skating, and short-track speed skating. These blood samples were tested at the venues by international sport federations before competition for hemoglobin concentrations and reticulocyte counts. If the reticulocyte count exceeded 2% or if the hemoglobin concentration exceeded 16.5 g% for women or 17.5 g% for men, the athlete returned after competition and an additional blood and urine samples were obtained for EPO analysis at the laboratory. Of the 1,222 blood samples, 133 (10.6%) had reticulocyte counts >2% and eight samples (0.6%) had increased hemoglobin concentrations [72]. In total, the laboratory received 77 combined blood and urine samples and of these, samples from three athletes met our criteria for darbepoetin alfa use [72]. All three athletes were cross-country skiers who had won a total of eight medals.

According to the procedure in effect at these Olympics, before announcing test results to the public, a committee reviews the laboratory findings and two hearings are held. The hearings, which are attended by the representatives of the athlete with or without the athlete, delves into the details of the testing. If the testing results are upheld, the IOC Executive Board reviews the case and makes the final decision to announce the findings to the public. In addition, the athlete has the right to appeal the IOC decision to CAS. As there is little to lose, most athletes do appeal to CAS. The darbepoetin alfa cases were be adjudicated by CAS in late 2002 and early 2003.

Epoetin alfa and darbepoetin alfa detection studies

Because athletes do not reveal their doses or dosing regimen, the only experimental approach to determine how to test for the occurrence of doping is to administer likely doses and analyze the urine at various times after the last dose. The early work of Wide et al. [65] established that their gel electrophoresis assay detected urinary rHuEPO for up to 24 hours after administering rHuEPO at 60 U/kg/week for seven to nine weeks.

We have used the isolectric focusing method [68–70] to determine the approximate time-course of detection of epoetin alfa in urine. We gave

150 U/kg/week to 15 healthy young volunteers for three weeks. If the hematocrit exceeded 48%, the next dose was withheld. After discontinuing epoetin alfa, all urine samples were positive by isoelectric focusing for two days and approximately 50% were positive for four days.

We have also determined that darbepoetin alfa is detectable in urine for up to 12 days. A modest single dose (0.40 μg/kg) was detected in eight of nine subjects for two to four days post-administration and in some subjects for up to 12 days post-administration. Thus, the detectability of darbepoetin alfa appears to be similar or longer than that of rHuEPO, which is consistent with darbepoetin alfa's longer serum half-life [73].

One limitation of detection-time investigations is that they do not necessarily mimic the dosage regimens used by athletes. Typically athletes become aware of the retrospectivity of a test and adjust their doping schedules so as to evade detection. In the case of a rapidly evolving new test, like isoelectric focusing for urinary rHuEPO, athletes are undoubtedly searching for ways to foil tests as diligently as laboratories are working on improvements to the tests. Perhaps the athletes who were found to be using darbepoetin alfa at the 2002 Games of Salt Lake did not expect the test to detect it.

Retrospectivity of the urinary isoelectric focusing test

The ability of isoelectric focusing to control the abuse of erythropoietic proteins among athletes is a complex function of dose, dosing regimen, detection times, urine collection time relative to last dose, and whether or not the test is announced in advance or a surprise (short-notice or out-of-competition testing). Sport administrators control the testing time and it is generally agreed that unannounced short-notice testing is the most effective deterrent. The athlete controls the dose and dosing interval, and the ability of the test to detect erythropoietic proteins is related to the urinary pharmacokinetics of the protein and the inherent sensitivity of the test. A good test is one that can detect the erythropoietic proteins for as long as the beneficial effects of the drug are present. The interplay of these factors is only beginning to be explored.

The pharmacodynamic effects of erythropoietic proteins that are particularly pertinent in the context of detecting users and that have been studied in healthy subjects, as opposed to patients with anemia, are the effects on hematocrit, hemoglobin concentration, and $VO_{2\,max}$. The effect on $VO_{2\,max}$ is the most relevant because it is a direct measure of performance. rHuEPO administered at doses of 150 to 230 U/kg/week for approximately three weeks produces an increase of $VO_{2\,max}$ of 6% to 8%, as expected [39–41]. Only two studies have monitored $VO_{2\,max}$ after rHuEPO was discontinued. In the first study, rHuEPO was administered for 25 days (150 U/kg/week), and four weeks after the last dose, the $VO_{2\,max}$ determined was not distinguishable from the $VO_{2\,max}$ of the placebo group [74]. It has been suggested that athletes may use a low-dose maintenance regimen that is sufficient to maintain the hematocrit just beneath the 50% health test threshold [40, 67]. To explore the effects of a low-dose regimen on $VO_{2\,max}$, Russell et al. [40] administered rHuEPO for three

weeks at a dose of 150 U/kg/week, then decreased the dose to 60 U/kg/week for an additional five weeks. They determined that the $VO_{2\,max}$ in week 12, four weeks after the last dose, was still increased (3.1% to 4.5%). The hematocrit remained increased for approximately 17 days after the last dose. Although these results need to be confirmed, the data on low doses of rHuEPO suggest that for a urine test for rHuEPO to be effective, it should be capable of detecting rHuEPO for three to four weeks after discontinuing a dose of 60 U/kg/week. Such data are not available.

Another approach to the retrospectivity issue is to assume that the effect on $VO_{2\,max}$ lasts as long as the hematocrit or hemoglobin concentration remain increased. While this assumption has not been directly investigated, it is reasonable to assume that the duration of action on $VO_{2\,max}$ will correlate with hematocrit and hemoglobin concentration. In the three studies [40, 41, 74] that measured hematocrits after rHuEPO was discontinued, a fixed dose of rHuEPO (80–230 U/kg/week) was administered for three to five weeks. During the rHuEPO administration phase, the hematocrit steadily increased until a plateau of approximately 50% was reached at 12 to 14 days. The hematocrit-time graphs of these studies show that the hematocrit is unchanged (approximately 50%) for 12 to 20 days after rHuEPO is discontinued. The hematocrit was last measured on days 24, 28, and 30 post-administration, and for each study the hematocrit was still greater than that of the placebo group. Thus performance as measured by $VO_{2\,max}$ and the hematocrit appear to follow more or less parallel time-courses, again consistent with three to four weeks of enhancement after the last dose. An athlete could be enhanced and yet have a negative urinary isoelectric focusing test.

Other erythropoietins

Athletes will experiment with other erythropoietic proteins as they become available. If laboratories are to keep up with this fast-moving field, they need detailed information on the new products, a reference standard for the product, and at a minimum, a urine sample collected from a subject known to be receiving the substance. Ideally, the laboratories would administer the substance to volunteers to obtain data on the isoform patterns and the pharmacokinetics of detection. These requirements are not easy to fulfill, particularly in the United States where ethics committees generally require that the drug be available as Food and Drug Administration (FDA)-approved product. If not, the investigator must file an application for a Investigational New Drug, a lengthy, complicated, and expensive process. Alternatively, the investigator may collaborate with colleagues who are working under an FDA-approved protocol.

Epoetin alfa, epoetin beta, and darbepoetin alfa

The current isoelectric focusing test [67] is able to detect administration of epoetin alfa, epoetin beta, and darbepoetin alfa [69, 70], allowing sport to con-

trol these erythropoietic proteins provided that the urine is collected when sufficient amounts of the material are present. More work is needed on the time-course of detection after various doses have been administered. A few studies are underway.

Epoetin omega

Epoetin omega has been administered to patients with anemia receiving hemodialysis [75] and some information is available on its structure [76]. Epoetin omega is produced in baby hamster kidney cell cultures and is less acidic than epoetin alfa or epoetin beta. In the current isoelectric focusing assay, a reference standard of epoetin omega migrates to the most basic area of the electropherogram. We recently analyzed a urine sample with isoforms consistent with epoetin omega, suggesting that it is being used by some athletes.

Epoetin delta

We are following the development of epoetin delta (dynepo), produced by human cells and being developed by Transkaryotic Therapies, Inc. Until we can obtain a reference standard of epoetin delta and a known positive urine sample, we will not know whether or not the existing isoelectric focusing assay will be able to detect it. Detection will depend in large part on how the manufacturer selects the final glycoprotein fractions. We anticipate that the band pattern will be basic, like epoetin alfa, and therefore detectable in the current assay.

Generics

Proprietary DNA vector technology has been used to develop a generic epoetin alfa [77]. Undoubtedly, new erythropoietic proteins are under development and products that are similar to epoetin alfa and epoetin beta are being produced outside the conventional pharmaceutical industry.

EPO *gene manipulation*

International sport organizations are mindful that gene manipulation might be used in the future to produce athletes with exceptional characteristics. At the first meeting of the IOC Gene Therapy Working Group, gene therapy was defined such that it would not include enhancement of athletes' performance ("...transfer of genetic material to human somatic cells for the treatment or prevention of disease or disorders") [78]. The group opined that gene manipulation is not on the immediate horizon, but that methods such as proteomic analysis might be developed to be prepared when gene-based doping becomes a reality.

Summary

The inherent complexity of the topic stems from interface between human behavior and the many disciplines that have something to offer, including chemistry, pharmacology, medicine, law, ethics, and sociology. Ultimately if sport continues to rely on testing as the solution, it is necessary to focus resources on physical and chemical methods for detecting drugs. At the same time, sport hopes to change its culture through education and espousing ethical positions.

Doping with erythropoietic proteins, the most recalcitrant and threatening problem for sport in recent years, is coming under control. The problem is threatening because the drugs are highly efficacious and they enhance performance in endurance sports and, perhaps, even sports that rely on short bursts of energy. The problem is recalcitrant because the methods to detect erythropoietic proteins in body fluids are complex, therefore global implementation is all the more difficult, and it is difficult to track athletes in their travels and implement collection procedures wherever they may be.

The current test is adequate from the perspective of unambiguous identification and legal defensibility, and it will no doubt improve; however, it is probably not sensitive enough to detect recombinant erythropoietic proteins for as long as performance enhancement will last. Fortunately such a degree of sensitivity is not required to contain the overall problem: The current test simply needs to be applied widely enough in short notice, out-of-competition testing.

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"Genetic Doping" with erythropoietin cDNA in primate muscle is detectable

Françoise Lasne,[1,*] Laurent Martin,[1] Jacques de Ceaurriz,[1] Thibaut Larcher,[2] Philippe Moullier,[2,3,*] and Pierre Chenuaud[2]

[1]*National Anti-Doping Laboratory, 92290 Chatenay-Malabry, France*
[2]*INSERM U 649, CHU Hotel-Dieu, and*
[3]*EFS Pays de Loire, 44035 Nantes, France*

[*]*To whom correspondence and reprint requests should be addressed. Laboratoire de Therapie Genique, Inserm U649, CHU Hotel-Dieu, Bat. J. Monet, 30 boulevard Jean Monnet, 44035 Nantes, Cedex 01, France*

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Forthcoming "genetic doping" is predicted to be undetectable. In the case of recombinant human erythropoietin (rhEPO), a hormone used in endurance sports, it is being predicted that exogenous drug injections will be replaced by the transfer of the corresponding gene into some of the athlete's own cells. The hormone thus produced inside the organism is assumed to be completely identical to the physiological one. Our results show that this is not the case and open up optimistic prospects for antidoping control involving gene transfer.

Doping in sport, with very few exceptions, arises from misused medical treatments. This is the case for rhEPO, a hormone that stimulates red blood cell production and that has become a key element of doping in endurance sports. Treatment with rhEPO currently requires repeated injections of recombinant hormones obtained from nonhuman cells, i.e., Chinese hamster ovary (CHO) and baby hamster kidney (BHK) cells, into which the human gene of the hormone has been inserted. Natural endogenous and rhEPO were shown to present different isoelectric profiles, probably the result of altered posttranslational modifications that are species- and tissue type-dependent. This difference has allowed for the development of a test to detect the presence of rhEPO in urine, a test that is currently used in antidoping controls [1].

Genetic technologies are expected to change the very nature of medical treatments. For instance, it is now conceivable that administration of an exogenous therapeutic protein will be replaced by introducing the corresponding gene into some of the patient's own cells. It is almost inevitable that athletes will exploit such medical progress in an effort to elude detection by sport authorities charged with curbing doping practices. Doping practices, in addition to being the focus of regulatory issues, may also severally and adversely affect the health of athletes that engage in such practices. Doping by gene transfer may compound these adverse side effects because of direct toxic effects, persistent gene expression, or potential insertional mutagenesis [2,3]. Furthermore, the assumption that these new methods of doping will yield proteins that are identical to the endogenous gene product, thus making detection impossible, may not be the case.

To compare the isoelectric profiles of physiological EPO and hormone resulting from *in vivo* gene transfer, we have adapted for serum analysis a method previously developed for urine [4]. Using this method, samples from cynomolgus macaques were analyzed for the serum recombinant EPO profile before and after transfer of the homologous cDNA into skeletal muscle by injection of recombinant adeno-associated virus [5]. Transgene expression was controlled by a doxycycline-regulatable system [6].

The physiological isoforms of the simian hormone were very similar to those of human urine EPO (Fig. 1b). Induction of transgene expression in these macaques resulted in overexpression of a hormone presenting a pattern strikingly different from that of the endogenous isoforms (Fig. 1c). The transgene-derived isoforms resolved with isoelectric focusing at higher pH, a finding more characteristic of recombinant EPO than endogenous EPO (Fig. 1a). In primates, EPO is primarily synthesized by renal peritubular fibroblasts [7]. The distinctive isoelectric pattern of recombinant EPO produced by skeletal muscle emphasizes the importance of cell type on the characteristics of recombinant EPO.

It is noteworthy that the structural features responsible for the described differences between the isoelectric patterns of physiological human urinary EPO and those of recombinant hormone are not yet clarified [4]. The newly observed differences in the macaque serum

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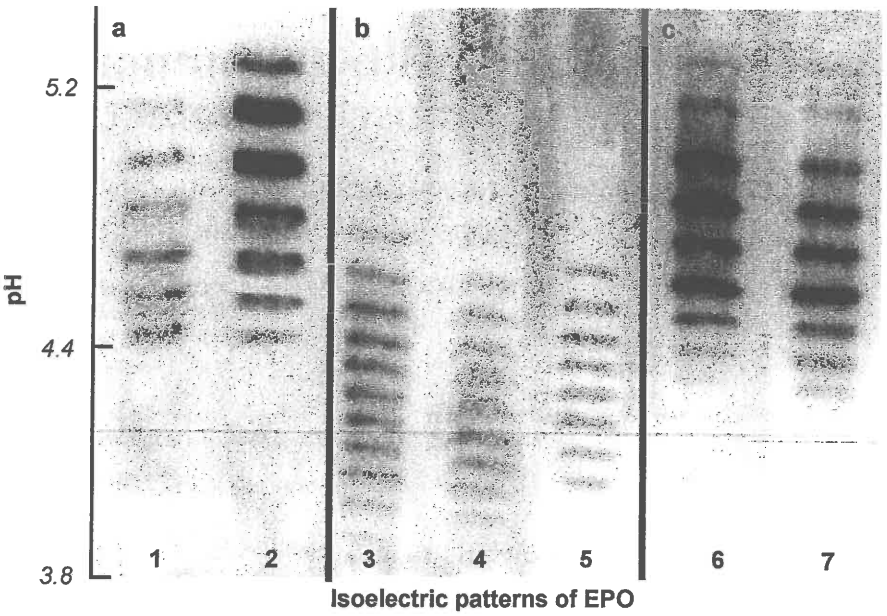

Fig. 1. Isoelectric patterns of erythropoietin. (a) rhEPO from CHO cells (lane 1) and BHK cells (lane 2). (b) Physiological EPO from human urine (lane 3) and macaque serum (lanes 4 and 5). (c) EPO from macaque serum after gene transfer in skeletal muscle (lanes 6 and 7). Serum samples (5) and (6) are from the same animal before and after gene transfer, respectively. Specific detection of EPO was obtained by double-blotting following isoelectric focusing. Cathode is at the top.

between the pattern of physiological EPO and that from transduced muscle are every bit as striking and require further study. Because a previous report [8] indicated that EPO extracted from serum was not as different in isoform distribution from recombinant EPO as was urinary EPO, the difference that we report here between the endogenous and the transgene-derived product from the serum samples is even more relevant. However, because the current test for rhEPO in sport uses urine, our study will have to be extended to this biological fluid.

The biological effects of recombinant EPO from genetically engineered muscle have been demonstrated in animal models [9,10]. However, our observations indicate that this recombinant EPO, like the other sources of rhEPO, is not identical to the physiological hormone. Skeletal muscle, since it is an easily accessible and efficiently transduced tissue, is likely to be the target tissue of choice for genetic doping. Although other methods of gene transfer exist and may be exploited for gene doping, and such methods are yet to be investigated, our results provide encouraging evidence that doping by gene transfer will likely not go undetected at least when skeletal muscle is the target.

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ARTICLE

Long-Term Doxycycline-Regulated Transgene Expression in the Retina of Nonhuman Primates Following Subretinal Injection of Recombinant AAV Vectors

Knut Stieger,[1] Guylène Le Meur,[1,2] Françoise Lasne,[3] Michel Weber,[1,2] Jack-Yves Deschamps,[4] Delphine Nivard,[1] Alexandra Mendes-Madeira,[1] Nathalie Provost,[1] Laurent Martin,[3] Philippe Moullier,[1,5] and Fabienne Rolling[1,*]

[1]*INSERM UMR U649, CHU-Hotel Dieu, Bât. J. Monnet, 30 Avenue J. Monnet, 44035 Nantes Cedex 01, France*
[2]*Service d'Ophtalmologie, CHU-Hotel Dieu, 1 Place Alexis Ricordeau, 44000 Nantes, France*
[3]*Laboratoire National de Dépistage du Dopage, 92290 Châtenay-Malabry, France*
[4]*Service d'Urgences, Ecole Nationale Vétérinaire, 44000 Nantes, France*
[5]*EFS-Pays de Loire, 44000 Nantes, France*

**To whom correspondence and reprint requests should be addressed. Fax: +33 2 40 08 74 91. E-mail: fabienne.rolling@univ-nantes.fr.*

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Adeno-associated viral gene therapy has shown promise for the treatment of inherited and acquired retinal disorders. In most applications, regulation of expression is a critical concern for both safety and efficacy. The purpose of our study was to evaluate the ability of the tetracycline-regulatable system to establish long-term transgene regulation in the retina of nonhuman primates. Three rAAV vectors expressing the tetracycline-dependent transactivator (rtTA) under the control of either the ubiquitous CAG promoter or the specific RPE65 promoter (AAV2/5.CAG.TetOn.epo, AAV2/4.CAG.TetOn.epo, and AAV2/4.RPE65.TetOn.epo) were generated and administered subretinally to seven macaques. We demonstrated that repeated inductions of transgene expression in the nonhuman primate retina can be achieved using a Tet-inducible system via rAAV vector administration over a long period (2.5 years). Maximum erythropoietin (EPO) secretion in the anterior chamber depends upon the rAAV serotype and the nature of the promoter driving rtTA expression. We observed that the EPO isoforms produced in the retina differ from one another based on the transduced cell type of origin within the retina and also differ from both the physiological EPO isoforms and the isoforms produced by AAV-transduced skeletal muscle.

Key Words: doxycycline-regulated transgene expression, retina, nonhuman primate, AAV vectors, erythropoietin

INTRODUCTION

Gene replacement therapy constitutes the most straightforward approach for treating autosomal recessive retinal disease. However, inherited retinal degenerations are triggered by mutations in a variety of genes [1]. Although the mechanisms by which the genetic defects lead to photoreceptor death are still not clear, the final common pathway is apoptosis [2]. Therefore, there is a major interest in developing a more generally applicable survival factor therapy that does not target the mutant gene product but rather alters the photoreceptor environment in a manner that promotes cell survival. Specific examples of such survival factors include neurotrophic factors that have the ability to modulate neuronal growth during development to maintain existing cells and to allow recovery of injured neuronal populations [3].

Recombinant AAV2-mediated gene transfer using neurotrophic factors such as fibroblast growth factor [4,5] and glial cell line-derived neurotrophic factor [6] have increased photoreceptor survival in different rodent models of retinal degeneration. Recombinant AAV2-mediated gene transfer of ciliary neurotrophic factor (CNTF) in an opsin$^{-/-}$ mouse prolonged photoreceptor survival following subretinal injection [7]. Interestingly, the same vector injected intravitreally in postnatal Prph2$^{Rd2/Rd2}$ mice and in adult P23H and S334ter rhodopsin transgenic rats showed prominent morphological protection of photoreceptors, but with no improved electroretinography (ERG) in the Prph2$^{Rd2/Rd2}$ mice and with lower ERG amplitudes in rat models [8]. Two recent reports clearly demonstrate that intraocular CNTF expression using rAAV2-mediated gene delivery, in

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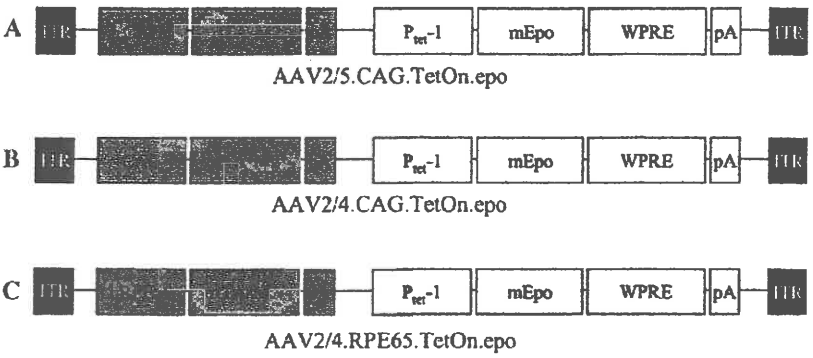

FIG. 1. Structure of rAAV vectors. Vectors encode the macaque erythropoietin cDNA (mEpo) under the control of the doxycycline-inducible TetO.CMV promoter and the rtTA chimeric transactivator (rtTA-M2) under the control of either the CAG promoter or the human RPE 65 promoter. pA (rtTA-M2), bovine growth hormone polyadenylation signal; pA (mEpo), SV40 polyadenylation signal; WPRE, woodchuck hepatitis virus posttranscriptional regulatory element; ITR, inverted terminal repeat of AAV2. Cassettes were incorporated into AAV4 or AAV5 capsids as indicated.

wild-type mice, in $Prph2^{Rd2/Rd2}$ mice, and in mice with a P216L rds/peripherin mutation, resulted in a significant decrease in function as assessed by ERG [9,10]. It is possible that CNTF has a narrow therapeutic window and that overproduction results in toxicity. Hence, a key issue for successful ocular gene therapy using such neurotrophic factors may be the ability to regulate transgene expression.

Several regulation systems, including rapamycin [11,12], mifepristone [13], ecdysone [14], and tetracycline (Tet) [15,16] have been developed to control transgene expression *in vivo*. Rapamycin-mediated regulation of erythropoietin (EPO) expression has been demonstrated in rats and nonhuman primates following intramuscular and subretinal administration of two rAAV vectors, one encoding the transactivator, the other EPO [17–19]. Although the rapamycin analog-regulatable system appears promising, it is yet a rather recent development in gene therapy and therefore likely deserves further pharmacological and toxicological studies.

The tetracycline-regulatable or its derivate the doxycycline-regulatable system is extremely attractive simply because the drug used to induce transcription is a well-known antibiotic, essentially devoid of major secondary effects. Doxycycline (Dox) has been extensively used in patients and its pharmacological and toxicological characteristics are known.

Dox has also proved to be efficient and reliable in controlling transgene expression *in vivo* following intramuscular injection of rAAV carrying the tetracycline transactivator (tTA) and EPO cDNA in mice [20,21] and primates [22,23]. In the retina, tetracycline-controlled expression of green fluorescent protein (GFP) in retinal pigmented epithelium (RPE) and photoreceptor cells has been demonstrated in rats following subretinal injection of the rAAV2 vector [24]. In a previous report, we showed that a single rAAV2 vector carrying the tTA and the GFP cDNAs can provide controlled transgene expression in rat ganglion cells after intravitreal injection and that repeated induction and regulation of the transgene was sustained over a 6-month period [25]. However, the current development of the Dox-regulatable system seems to be doomed simply because when translated in skeletal muscle of nonhuman primates, the Dox-sensitive tetracycline-dependent transactivator (rtTA) is eventually recognized by the immune system in 80% of the primates challenged with such a system [22,26].

In this study, however, we demonstrate the ability of the tetracycline-regulatable system to establish long-term transgene regulation in the retina of nonhuman primates following subretinal injection of both AAV vectors type 4 and type 5. EPO was chosen as a "reporter gene" because it is a secreted protein and expression levels can easily be quantified. Since previous studies reported abnormal posttranslational modifications of transgene products

TABLE 1: List of primates and AAV doses injected

Macaque	Vector	Vector titer (vg/ml)	Volume (μl)	EPO concentration in a.c.f. (mU/ml) Baseline before induction	Peak 1	Duration of study (mpi)
P5.1	AAV2/5.CAG.TetOn.epo	1×10^{12}	100	45	1662[a]	28
P5.2 (Mac1)	AAV2/5.CAG.TetOn.epo	5×10^{11}	100	26	760	9
P5.3L	AAV2/5.CAG.TetOn.epo	2×10^{11}	100	14	423	9
P5.3R	AAV2/5.CAG.TetOn.epo	2×10^{11}	150	37	584	9
P4.1 (Mac2)	AAV2/4.CAG.TetOn.epo	2×10^{11}	100	5	99	9.5
P4.2	AAV2/4.CAG.TetOn.epo	2×10^{11}	90	6	75	9.5
P4.3	AAV2/4.hRPE65.TetOn.epo	2×10^{11}	100	1	14	12.5
P4.4	AAV2/4.hRPE65.TetOn.epo	2×10^{11}	100	0	13	12.5

EPO concentrations are shown before and during the first induction. vg, vector genome; a.c.f., anterior chamber fluid; mpi, months postinjection.
[a] EPO concentration measured in the vitreous.

when expressed from an ectopic site [27,28] with possible adverse effects [26], we compared isoelectric profiles of retinal-derived EPO with both physiological EPO isoforms and the isoforms produced by AAV-transduced muscle.

Results

Vector Design

We generated rAAV vectors of different serotypes containing the macaque EPO cDNA and the rtTA driven by either the CAG or the RPE65 promoter. We have previously demonstrated that, following subretinal injection in dogs and nonhuman primates, rAAV2/4 leads to transduction exclusively of the RPE [29], in contrast to rAAV2/5, which transduces both the RPE and photoreceptors [29,30]. Therefore we produced three different vectors: AAV2/5.CAG.TetOn.epo and AAV2/4.CAG.TetOn.epo vectors, in which the CAG promoter controls the rtTA expression (Figs. 1A and 1B), and rAAV2/4.RPE65.TetOn.epo vector, in which the RPE65-specific promoter controls rtTA expression (Fig. 1C).

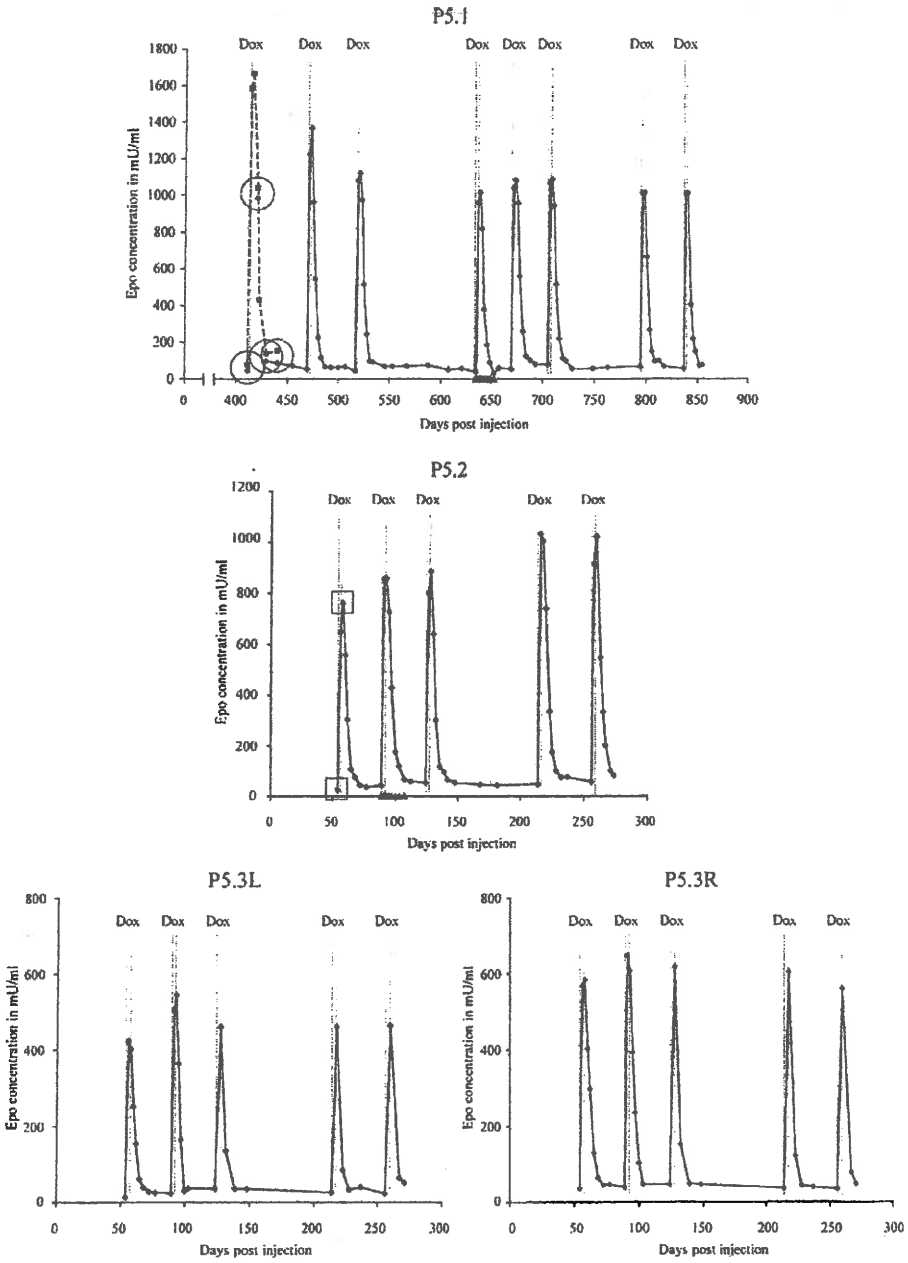

FIG. 2. Time course of EPO concentration in the anterior chamber fluid or vitreous of three nonhuman primates after subretinal delivery of AAV2/5.CAG.TetOn.epo. Three-day induction cycles are indicated as "Dox." For macaque P5.1, EPO concentrations in the anterior chamber fluid and vitreous of the injected eye were compared (indicated by a circle). For macaque 5.2, squares indicate time points for which IEF patterns of EPO were evaluated (see Figs. 5A and 5B). For macaques P5.1 and P5.2: (♦) anterior chamber fluid right eye (injected), (■) vitreous right eye (injected), (▲) anterior chamber fluid left eye (uninjected control).

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We treated a total of seven macaques: P5.1 through P5.3 with rAAV2/5 and P4.1 through P4.4 with rAAV2/4 (Table 1). We injected all the macaques unilaterally in the right retina except for P5.3, which we treated in both eyes (P5.3L and P5.3R).

Long-Term Regulation of EPO Expression in Macaques Following Subretinal Injection of the AAV2/5.CAG.TetOn.epo Vector

Cycles of Dox induction of the AAV2/5.CAG.TetOn.epo vector-treated macaques started at 8 weeks postinjection except for the pilot macaque (P5.1), in which the first induction occurred at 58 weeks postinjection (Fig. 2).

Since the vitreous fluid, adjacent to the retina, connects to the anterior chamber fluid, diffusion of EPO through the vitreous into the anterior chamber fluid allowed for repeated sampling of protein levels in the same animal. To compare the concentration of EPO in the vitreous and in the anterior chamber fluids, we sampled both compartments during the first induction of P5.1 (circles in Fig. 2). As expected, we found similar levels of EPO in both compartments (at 8 days post-induction for example, 1042 mU/ml in the vitreous and 983 mU/ml in the anterior chamber fluid). Since sampling the anterior chamber fluid is less invasive than sampling the vitreous fluid, we punctured only the anterior chamber fluid for all other time points and animals.

All AAV2/5.CAG.TetOn.epo-treated retinas showed tightly regulated EPO synthesis, over a 2-year period for P5.1 and over an 8-month period for P5.2 and P5.3 (duration of the experiment). In all macaques, the peak of EPO production consistently occurred at 48 h post-Dox induction and returned to baseline level within 10 days upon the withdrawal of Dox (Fig. 2). The peak levels obtained showed a tight correlation between the amount of AAV2/5.CAG.TetOn.epo injected and the transgene expression. Macaque P5.2, which received 2 times fewer particles than P5.1, displayed a 2.2 times lower level of EPO at the first induction. Indeed, 2.5 times fewer AAV2/5 vector genomes injected into P5.3L versus P5.2 resulted in a 2 times lower EPO concentration. Similarly, the 1.5 times fewer particles injected into P5.3R versus P5.2 resulted in 1.4 times lower EPO concentration. Another relevant finding was the tight control of the transgene expression during the off state (−Dox). In the on state, all animals expressed 20 to 30 times the baseline EPO level. For P5.1 and P5.2, we collected the anterior chamber fluid of the contralateral eye and analyzed it during the fourth or the second induction, respectively (triangles in Fig. 2).

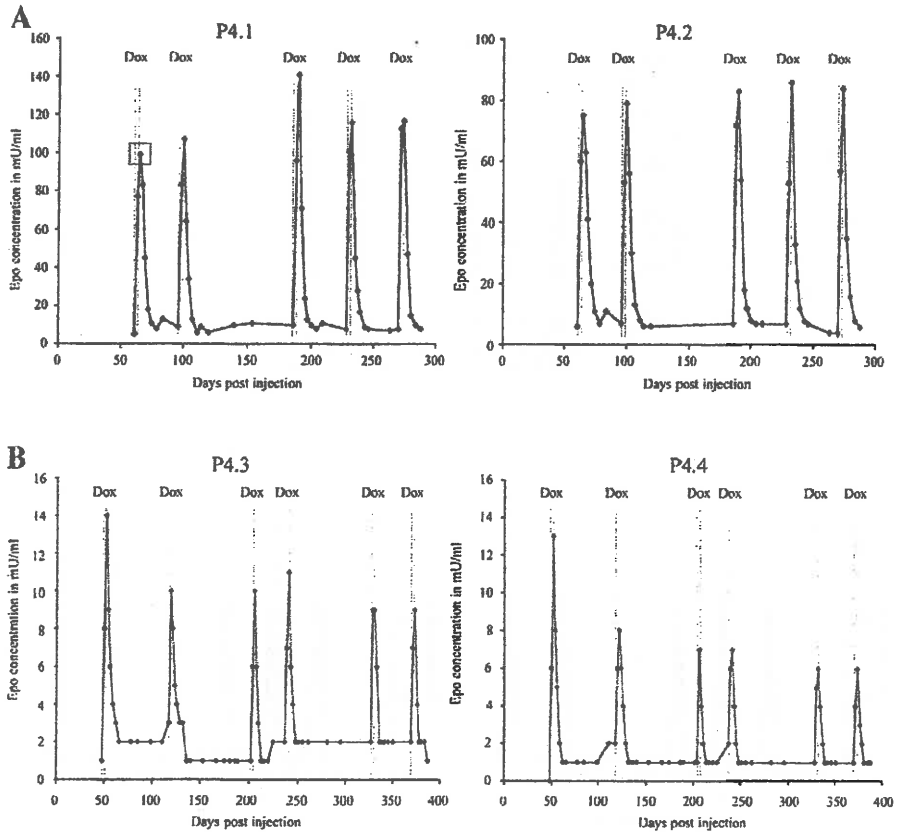

FIG. 3. Time course of EPO concentration in the anterior chamber fluid of four nonhuman primates after subretinal delivery of AAV2/4.CAG.TetOn.epo and AAV2/4.RPE65.TetOn.epo vectors. Three-day induction cycles are indicated as "Dox." A square indicates the time point at which IEF patterns of EPO were evaluated (see Fig. 5C). (A) AAV2/4.CAG.TetOn.epo subretinally injected macaques P4.1 and P4.2. (B) AAV2/4.RPE65.TetOn.epo subretinally injected macaque P4.3 and P4.4.

EPO was never detected in the anterior chamber fluid of the uninjected contralateral eye.

Long-Term Regulation of EPO Expression in Macaques Following Subretinal Injection of AAV2/4.CAG.TetOn.epo and AAV2/4.RPE65.TetOn.epo Vectors

We injected four macaques (P4.1 through P4.4) with AAV2/4.CAG.TetOn.epo (n = 2) and AAV2/4.RPE65.TetOn.epo (n = 2) (Table 1). Cycles of Dox induction started at 8 weeks postinjection in all macaques. Upon Dox induction and withdrawal, all four AAV2/4-injected primates showed similar EPO expression profiles compared to the AAV2/5 animals, albeit with lower resultant EPO concentrations (Fig. 3). Following injection of the same amount of vector genomes, AAV2/4.CAG.TetOn.epo-treated macaques (P4.1 and P4.2) displayed 4 times lower EPO concentrations than AAV2/5.CAG.TetOn.epo-treated macaques. This result can be explained by the fact that, given the tropism of the different AAV serotypes, in AAV2/4-injected primates, EPO is produced exclusively by the RPE, while in AAV2/5-treated animals both the RPE and the photoreceptors secrete EPO. Interestingly, when control of the rtTA expression is under the RPE65 promoter (P4.3 and P4.4) versus the CAG promoter, there is a 10-fold decrease in EPO concentration. This result shows the difference of relative strength between the activity of the CAG and the RPE65 promoters in macaque RPE cells *in vivo*. Finally, in both AAV2/4 vector constructs, the level of EPO expression in the on state was 15 to 20 times the baseline EPO level.

Assessment of Retinal Morphology and Retinal Function Following Long-Term Tet-Regulated EPO Expression

Optical coherence tomography (OCT) is a noncontact, noninvasive optical imaging technique that measures the intensity of back-scattered light [31,32]. OCT produces

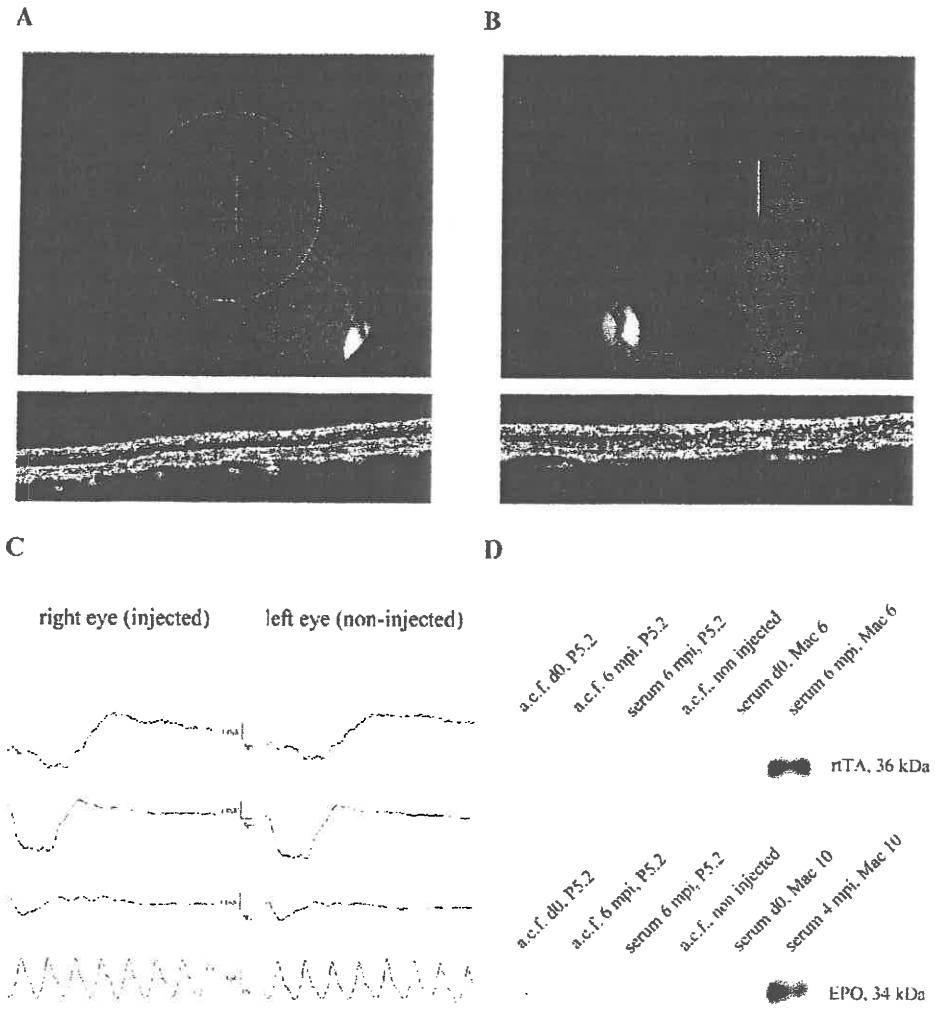

FIG. 4. Assessment of retinal morphology and function following long-term Tet-regulated EPO expression and evaluation of humoral immune response against the rtTA-M2 and EPO proteins. (A) Fundus photography and OCT image of the right eye of macaque 5.2 (injected). The green line indicates the scanning path. The white dotted circle indicates the zone of the bleb. (B) Fundus photography and OCT image of the left eye of macaque 5.2 (uninjected control). The green line indicates the scanning area of the retina. (C) Standard ERG recordings of both eyes of macaque 5.2. Graphs correspond to rod ERG, maximum ERG, cone ERG, and flicker (from the top to the bottom line). (D) Evaluation of antibody generation against rtTA-M2 and EPO by Western blot analysis. a.c.f., anterior chamber fluid; mpi, months postinjection.

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cross-sectional images of optical reflectivity in the tissue analogous to an ultrasound B-scan, but providing greater resolution by using light instead of sound waves. We performed OCT imaging in all treated animals to monitor subsequent recovery from the initial subretinal bleb and the morphology of the retina after long-term expression of rtTA and several inductions of EPO expression. Long-term OCT monitoring documented preservation of the retinal thickness in both treated and untreated retinas, in all animals (Figs. 4A and 4B).

We evaluated the effects of long-term rtTA and EPO expression on global retinal function in macaques using flash photopic and scotopic ERG. The same investigator (G.L.) recorded the ERGs in a standardized fashion up to 24 months p.i. for P5.1 and 8 months for P5.3 and P4.1–P4.4. Normal patterns of ERG amplitudes were elicited in both the treated and the untreated eye of all seven animals studied. As an example, the ERG recordings of P5.2 are presented in Fig. 4C.

Anterior chamber fluid and serum were evaluated for the presence of antibodies against rtTA and EPO. As assessed by Western blot, none of the animals developed antibodies against these two proteins (Fig. 4D).

Isoelectric Profile of Transgene-Derived Erythropoietin is Cell Type Dependent in Macaques

We evaluated the isoelectric profiles of the secreted EPO of all treated macaques from various time points follow-

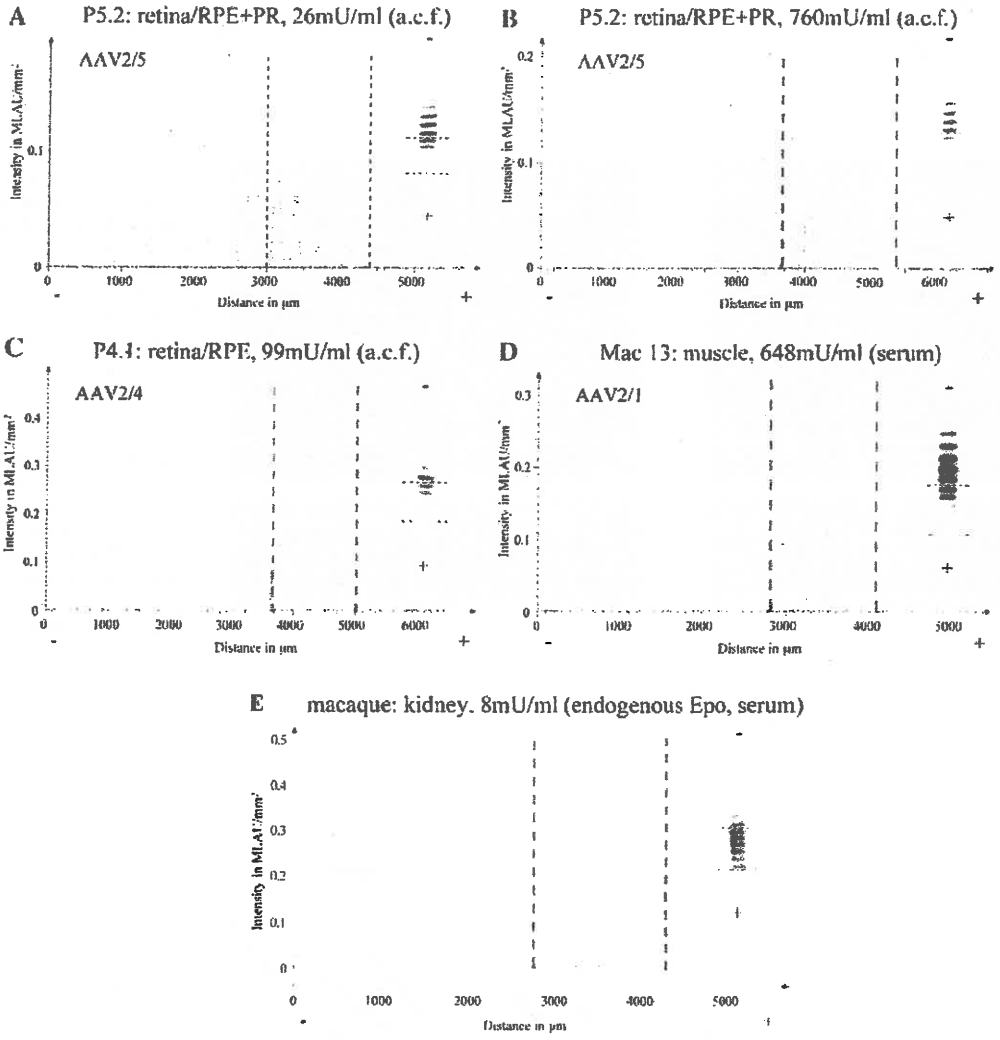

FIG. 5. Isoelectric focusing patterns and corresponding integrated profiles of EPO. Fluorescence intensity is reported in mega linear arbitrary units (MLAU). (A, B) AAV2/5.CAG.TetOn.epo subretinally injected macaque P5.1. (C) AAV2/4.CAG.TetOn.epo subretinally injected macaque P4.1. (D) AAV2/1.CAG.TetOn.epo intramuscularly injected primate (Mac13 in [23]). (E) Uninjected macaque.

ing the first Dox induction. As observed for P5.2 (time points are presented in Fig. 1 as squares), the isoelectric focusing (IEF) patterns of EPO from the aqueous humor were identical in both the uninduced (26 mU/ml) and the induced (760 mU/ml) state, for all individuals. Another observation is that secreted EPO of the AAV2/5 (P5.2) and AAV2/4 (P4.1) subretinally injected primates displayed differing IEF patterns (Figs. 5A–5C). Moreover, the patterns were different from those of physiological EPO isoforms (Fig. 5E) or from isoforms produced by AAV-transduced skeletal muscle (Fig. 5D).

Discussion

In this study, we demonstrated that a single subretinal injection into macaques of an AAV2/4 or −2/5 vector containing a transgene under a tetracycline-dependent expression system results in an effective regulation of gene expression in the retina for at least 2.5 years. Maximum EPO secretion in the anterior chamber depends on the rAAV serotype used and the nature of the promoter driving rtTA expression.

All (100%) treated macaques displayed tightly regulated EPO synthesis with similar induction kinetics. The peak of EPO production consistently occurred at 48 h after Dox induction, returning to baseline level within 10 days upon withdrawal of Dox. The peak EPO levels obtained were 1000, 100, and 10 mU/ml for macaques injected with AAV2/5.CAG.TetOn.epo, AAV2/4.CAG.TetOn.epo, and AAV2/4.RPE65.TetOn.epo, respectively. Although the regulation was fairly tight in all seven macaques, AAV2/5.CAG.TetOn.epo-injected primates displayed leaky expression in the uninduced state (14 to 45 mU/ml) that exceeded levels of induced EPO expression in the AAV2/4.RPE65.TetOn.epo animals (13 to 14 mU/ml).

The 1-log difference of expression between AAV2/5.CAG.TetOn.epo and AAV2/4.CAG.TetOn.epo can be explained by the fact that, in AAV2/4-injected primates, EPO is exclusively produced by the RPE, while in AAV2/5-treated animals both the RPE and the photoreceptors are transduced [29]. This explanation is in agreement with the 20:1 mean ratio of photoreceptors to RPE in the macaque retina [33]. In contrast, the 1-log difference of EPO expression between AAV2/4.CAG.TetOn.epo- and AAV2/4.RPE65.TetOn.epo-treated animals is likely to be due to the strength of the promoter that drives the rtTA transactivator (CAG vs RPE65). This result suggests that the RPE65-specific promoter is 10 times less active than the CMV-derived promoter in primate RPE cells *in vivo*.

We previously reported that no vector genomes could be detected in the contralateral eye following subretinal injection in dogs and primates [34]. Consistent with this previous observation, in this study we were unable to detect EPO in the contralateral untreated eye.

An important finding in the present study is the long-term persistence of Dox-mediated transgene inducibility in 100% of the primates. Indeed, we previously reported that, using a similar AAVTetOn construct administered to the muscle via an AAV vector, iterative Dox inductions resulted in the complete loss of EPO secretion in 80% of the primates after two or three challenges [22]. The mechanism involved was a humoral and cytotoxic T cell response directed against the rtTA transactivator protein resulting in the destruction of the genetically modified muscle fibers. The fact that no antibody against rtTA or EPO could be detected in the anterior chamber fluid or in the serum, as assessed by Western blot analysis, and that 100% of macaques injected in the retina displayed persistent regulation of EPO secretion is consistent with the retina's status as an immune-privileged tissue. The Dox-regulatable system appears to be consistently and permanently functional in the nonhuman primate retina, suggesting that this could also be the case in patients. To our knowledge, this is the first demonstration that a therapeutic transgene can be tightly regulated using a safe and fully characterized drug essentially devoid of secondary effects. In our opinion, the only currently available alternative for clinical use is the recently described rapamycin-regulatable system using nonimmunosuppressive analogs [17]. Studies using rapamycin-inducible dual-expression vectors describe successful regulated gene expression in the retina of macaques [18,19]. The kinetics of induction and de-induction are different, however, from that of the Dox-regulated system. EPO expression in the anterior chamber fluid peaked 7–14 days postdelivery (2 days with the Dox-inducible system) and took several weeks to return to baseline (10 days with the Dox-inducible system) [19]. Moreover, expression levels never returned to baseline but stayed at slightly elevated levels after each induction in two of four subretinally injected eyes.

The IEF patterns of EPO from the aqueous humor of all individuals were identical in both the uninduced and the induced state. This observation suggests that in contrast to what has been previously suggested for highly expressed myotube-derived factor IX [28], overexpression of EPO in the retina was not a contributing factor for posttranslational modifications of EPO. Another observation made in the present study is that the secreted EPO of the AAV2/5 (RPE and photoreceptors) and AAV2/4 (RPE only) subretinally injected primates displayed differing IEF patterns and were both different from physiological EPO. The most consistent explanation for this is that posttranslational modifications of transgene-derived EPO in the retina differ with respect to the transduced cell type of origin. The retina-derived EPO isoforms are distinctive from the recombinant EPO produced by skeletal muscle [27]. This difference could explain the observed discrepancy between intramuscular and intraocular administration of AAV.epo with respect to the

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subsequent rescue of retinal function in animal models of retinitis pigmentosa [35]. It is possible that a specific isoform secreted from transduced muscle, but not in the transduced retina, may act directly on the EPO receptors of the retina. Further investigations will be needed to identify the functional isoform(s) involved.

In conclusion, we have developed and validated, in primate retina, stringent and long-term Dox-regulated transgene expression using the delivery of a single AAV2/5 or AAV2/4 vector. Three different ranges of EPO concentrations were achieved depending on the rAAV serotype and the nature of the promoter driving rtTA expression. We observed that recombinant EPO produced in the retina has distinctive characteristics depending on the transduced cell type and is different from the physiological EPO and the EPO produced by transduced muscle. This type of complication may also occur with other proteins, particularly when subject to a high degree of posttranslational modification, suggesting that this new parameter should be taken in account when designing and evaluating gene therapy protocols.

Materials and Methods

Recombinant AAV-TetOnEPO vectors. Generation of expression cassettes encoding EPO (P_{tet}-1-mEpo-WPRE-pA) and the transactivator rtTA-M2 under the control of the CAG promoter (CAG-rtTA-M2-pA) was previously described [23]. The expression cassette RPE65-rtTA-M2-pA was produced by replacing the 1149-bp CAG promoter fragment with the 806-bp human RPE65-specific promoter fragment (kindly provided by Robin Ali, London, UK). Two different vector plasmids flanked by the two AAV2 ITRs were constructed. The first vector plasmid, CAG-rtTA-M2-pA/P_{tet}-1-mEpo-WPRE-pA, was encapsidated into AAV5 and AAV4 shells, while the second vector plasmid, RPE65-rtTA-M2-pA/P_{tet}-1-mEpo-WPRE-pA, was encapsidated only into AAV4. Thus, three different vectors were produced, AAV2/5.CAG.TetOn.Epo, AAV2/4.CAG.TetOn.Epo, and AAV2/4.RPE65.TetOn.Epo (Fig. 1), as previously described [36]. The rAAV titers were determined by dot blot and expressed as vector genomes(vg)/ml [37].

Subretinal injection of rAAV vectors and Dox administration. Primates were purchased from BioPrim, Baziège, France. All animals were cared for in accordance with the ARVO statement for the use of animals in ophthalmic and vision research. Subretinal injections were performed via a transvitreal approach under isoflurane gas anesthesia as described previously [29]. Briefly, after a vitrectomy had been performed, a 44-gauge cannula was inserted through a sclerotomy and advanced through the vitreous. Under microscopic control, vector suspension was injected into the subretinal space underlying the central tapetal retina using a viscous fluid injection system (DORC International, The Netherlands). Volumes injected were between 90 and 150 µl and vector titers were 2×10^{11} vg/ml for AAV-2/4 and 5×10^{11} and 1×10^{12} vg/ml for AAV-2/5 (Table 1). The protocol was approved by the Institutional Animal Care and Use Committee of the University of Nantes. All subretinal injections were performed unilaterally on the right eye (except for macaque P5.3, who was injected into both eyes).

Induction of EPO expression was initiated 2 months after vector administration (except for macaque P5.1) and induction cycles consisted of a 3-day Dox pulse (doxycycline-Ratiopharm SF; Ratiopharm; 10 mg/kg) given intravenously and repeated every 1 or 2 months.

In vivo transgene regulation analysis. Sampling of the anterior chamber fluid and the vitreous was performed as follows. Primates were anesthetized with an im injection of ketamine (Imalgène; Rhone Merieux, France) and Medetomidin (Domitor; Pfizer). For sampling the anterior chamber fluid, a 27-gauge epicranial cannula was used. For sampling the vitreous, a 30-gauge cannula connected to a 1-ml syringe was used.

EPO concentration in the anterior chamber fluid and vitreous was measured by enzyme-linked immunosorbent assay (Quantikine IVD; R and D Systems) and reported in mU/ml.

Humoral immune response against rtTA-M2 and EPO. Detection of anti-rtTA-M2 and anti-EPO antibodies was performed using Western blot analysis as recently described [22]. Briefly, recombinant rtTA-M2 protein (240 ng, kindly provided by Hermann Bujard, Heidelberg) or recombinant human EPO (504 ng, NeoRecormon; Roche) was subjected to SDS–PAGE and transferred to a Hybond ECL nitrocellulose membrane (Amersham) for subsequent immunoblotting. Anterior chamber fluid (1/400) and sera (1/200) from our experimental macaques were tested for the presence of antibodies. Sera from macaques that have previously been shown to contain antibodies against rtTA-M2 and EPO were used as a positive control [22,26] and anterior chamber fluid from an uninjected macaque as a negative control. Incubation with sera and anterior chamber fluids was performed overnight at 4°C. Peroxidase-conjugated goat anti-rhesus monkey IgG (Southern Biotechnology Associates, Birmingham, AL, USA) was used as secondary antibody (1/2000, 2 h at 4°C) followed by enhanced chemiluminescence detection.

Fundoscopy. We monitored retinal morphology in our experimental macaques with fundus photography using a Canon UVI retinal camera (Lheritier SA, Saint-Ouen-l'Aumône, France) connected to a digital imaging system (Lledioph Win software; Lheritier SA) as previously described [38]. Briefly, pupils of the animals were dilated 20 min before anesthesia using tropicamide (Ciba Vision Faure; Novartis, France) and phenylephrine hydrochloride (10% Neosynephrine; Novartis) and anesthesia was given by intramuscular delivery of agents as described above.

Electroretinography. Retinal function was tested using simultaneous bilateral flash photopic and scotopic ERG using a computer-based system (Neuropack m MEB-9102K; Nihon-Kohden, Tokyo, Japan) and contact lens electrodes (ERGjet; Universo Plastic SA, Switzerland) as described previously [38]. Dilatation of the pupils was performed as described above and animals were kept under isoflurane gas anesthesia. We recorded ERGs in a standardized fashion, according to International Society for Clinical Electrophysiology of Vision protocols. Each contralateral eye served as uninjected control.

Optical coherence tomography. Retinal morphology was assessed by optical coherence tomography (Stratus 3000; Zeiss, Germany). Dilatation of the pupils and intramuscular anesthesia of the animals were performed as described above. Examination was performed by doing a 3-mm scan in the area of the bleb in the injected eye and the corresponding area of the contralateral, uninjected eye.

Isoelectric focusing and immunoblotting of EPO isoforms. To evaluate the isoelectric profiles of EPO, an isoelectric focusing method combined with a double immunoblotting technique previously developed for urine and serum was adapted for the anterior chamber fluid [27,39,40]. Briefly, after a prefocusing step at 250 V for 30 min, 20 µl of samples was applied to a polyacrylamide gel and subjected to IEF using the Multiphor II electrophoresis system (Amersham-Pharmacia) at 1 W/cm. Migration width was 9 cm. The run was stopped at 4000 Vh. After the IEF run, proteins were transferred to an Immobilon-P membrane for subsequent immunoblotting. The membrane was then washed, blocked, and incubated with the primary monoclonal anti-EPO antibody (1/1000). To prevent nonspecific binding of the secondary antibody, the membrane was subjected to a second blotting step. After the second transfer to an Immobilon-P membrane, incubation with a secondary biotinylated anti-mouse IgG antibody (1/4000) was performed followed by enhanced chemiluminescence detection using a charge-coupled device camera (Fuji, Japan). Profiles corresponding to the isoelectric patterns were obtained using AIDA 1D-Evaluation software from Fuji.

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Blood Doping

The effects of microdose recombinant uman erythropoietin regimens in athletes

This study appraised the veracity of claims that athletes can evade doping controls by injecting *microdoses* of recombinant human erythropoietin (rHuEPO), which rapidly disappear from the circulation. We confirmed that microdosing can reduce the window of detection to as little as 12-18 hours post-injection, suggesting that authorities must adopt appropriate counter measures.

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Athletes can illicitly use rHuEPO to boost red cell mass and thereby oxygen carrying capacity and endurance performance.[1] There are persistent suggestions that athletes have learnt to use rHuEPO, but test negative, by titrating rHuEPO dosage regimens in order to minimize the appearance of basic isoforms in urine samples (rHuEPO can be detected via electrophoresis because rHuEPO isoforms are more basic than endogenous erythropoeitin isoforms).[2]

It is vital for antidoping agencies to determine whether existing deterrent strategies have been circumvented. To establish whether it is possible to confound detection strategies by titrating rHuEPO dosages, our study simulated a so-called 'microdose' rHuEPO regimen and measured the level of basic isoforms in urine collected during and after the administration protocol.

Two well-trained male subjects (28 years old, 74 kg, 176.5 cm, regional level triathlete; 31 years old, 62 kg, 170 cm, national level long distance runner) gave informed consent to participate in this study which was reviewed and approved by the Regional Ethics Committee. Initially red cell production was rapidly accelerated in both subjects using high doses of rHuEPO (~260 IU/kg injections on days 0, 2, 4, 7, 9 and 11) in conjunction with a single intravenous iron treatment (100 mg), with the goal to elevate hemoglobin (Hb) concentration to approximately 170 g/L.

Over the next three weeks, injections were given every 2-3 days (injections on days 15, 17, 19, 22, 24, 26, 29, 31 and 33) and dosages were adjusted by a pharmacologist guided only by basic hematologic information (blood and reticulocyte counts, no urine profiles were provided as feedback). Microdosages were less than 10% of the initial dose (exact dosage undisclosed to prevent replication by athletes). Urine samples were collected three times per day during the microdose phase (7-9h, 11-13h, 19-21h), and analyzed for the presence of rHuEPO at the French national antidoping laboratory (Laboratoire National de Dépistage du Dopage, Paris).

As expected high dose rHuEPO treatment rapidly elevated Hb concentrations within ~2 weeks (140 to 166 g/L; 148 to 174 g/L; subjects 1 and 2, respectively). We found that it was possible to maintain elevated Hb values using microdoses of rHuEPO. After 3 weeks of the microdose regimen Hb concentrations were still 164 g/L and 170 g/L respectively (and 164 g/L and 162 g/L 1 week after all injections ceased). During the microdose phase reticulocyte percentages ranged in value

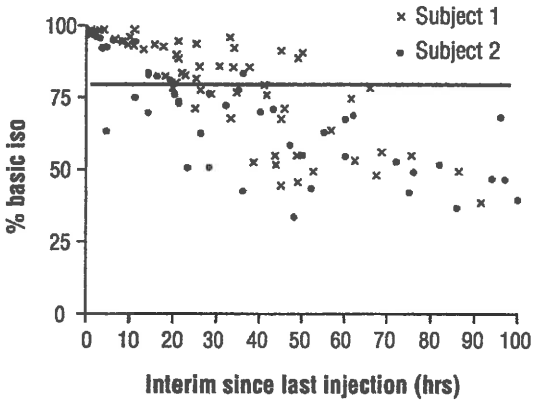

Figure 1. The percentage of basic isoforms in urine samples collected at various intervals after each of the nine *microdose* injections of rHuEPO. Injections were given over a three week period during which time both subjects maintained an elevated haemoglobin concentration of approximately 170 g/L. The horizontal line depicts an 80% threshold level that has been used previously to declare a sample positive for the presence of rHuEPO.

from 0.8-1.2% and 0.4-1.1% for the two subjects. Urine samples collected more than 24 hours after a microdose injection typically had less than 80% basic isoforms, which until recently was the criterion used to declare a sample positive (Figure 1).

In some instances samples collected just 12-18 hours after the last injection fell below the 80% threshold. It is noteworthy that our pharmacologist was able to quickly devise an effective microdose regimen utilizing limited feedback and with few prior attempts.

Interestingly isoelectric profiles showed the reappearance of endogenous erythropoietin bands during the microdose phase (*results not shown*). This is in contrast to the existing paradigm which holds that endogenous erythropoietin production is suppressed when the red cell mass has been increased beyond the homeostatic set point.

The implications of this remain unclear, however it can be speculated that were an athlete to receive microdoses of rHuEPO for an extended period (>2-4 weeks), it is conceivable that reappearance of endogenous bands of erythropoietin would be of sufficient magnitude to further reduce the effective window of detection of the test for rHuEPO.

Our results show that it is conceivable for athletes to maintain illicit rHuEPO doping even during multiday endurance events when competitors may be tested at the end of each day (ie at 24 hour intervals). The electrophoretic test has proven legally defensible and remarkably robust.

The recent adoption of improved detection criteria further enhances the discriminatory capacity of the urine test,[3] although whether this carries over to microdose samples awaits further research. The fact that microdoses of rHuEPO disappear rapidly from the circulation could be exploited by athletes to evade detection. This implies that authorities should supplement the urine test with an approach providing greater reach-back. This research also sharpens awareness that to be efficient, urine tests should be based on out of competition testing.

Michael Ashenden, Emmanuelle Varlet-Marie,° Françoise Lasne,* Michel Audran°*

**Science and Industry Against Blood doping (SIAB) research consortium, Gold Coast, Australia; °Biophysical & Bioanalysis Laboratory, Faculty of Pharmacy, University Montpellier I, Montpellier, France; #National Antidoping Laboratory, Châtenay Malabry, France*

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Correspondence: Michael Ashenden, Science and Industry Against Blood doping (SIAB) research consortium, Gold Coast, Australia. E-mail: heyasho@hotmail.com

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Letter to the Editor

New urinary EPO drug testing method using two-dimensional gel electrophoresis

Dear editor,

Two-dimensional gel electrophoresis (2D) of urine samples prepared by acetonitrile precipitation is presented by the authors as a reliable method to detect the presence of recombinant EPO in urine [1]. Many references are made to the current one-dimensional (1D) IEF based urinary EPO test used in anti-doping control and imply that this latter method may be unreliable. On the assumption that the same monoclonal anti-EPO antibody (AE7A5) was used in the 2 methods, it is speculated that the non-specific binding observed in the 2D method may affect the results of the 1D method.

It is very surprising that the authors, who have not tested the 1D method, have extrapolated some problems observed with *their* method to another one without showing any comparative results to support their statements. Yet it is well known that the specificity of antibodies may be heavily affected by the experimental conditions. In particular, the denaturation of proteins both by the treatment of urine (precipitation, reduction–alkylation before isoelectric focusing) and the SDS electrophoresis, as performed in the 2D method, exposes buried domains that may then react unspecifically with antibodies. Whatever the causes for non-specific binding in the 2D method, the specificity of the AE7A5 antibody has been, of course, thoroughly investigated in the 1D system. As demonstrated by the total absence of any bands in the window used for interpretation of the isoelectric profiles, in the case of samples devoid of EPO, no cross reaction occurs. The intensity of the image inside this window is always consistent with the EPO level in the retentate applied onto the IEF gel. Since the ELISA used for quantitative estimation of EPO in retentates involves a capture antibody and a detecting antibody that are both different from the AE7A5 used for blotting, the isoforms used for the interpretation of a result are identified as EPO by three different antibodies. The only signal not related to EPO (and not detected by ELISA) appearing with some retentates is observed outside the window and cannot interfere with the result of the analysis. Unlike the results of the 2D method, no interference with α-1-anti-chymotrypsin, α-2-thiol proteinase inhibitor, α-2-HS glycoprotein precursor, the pI of which overlap with the pI of rHuEPO (which is not the case of Tamm–Horsfall glycoprotein), actually occurs in the 1D method (Fig. 1). High concentrations of these proteins largely exceeding their usual levels in urine don't give rise to any bands. In conclusion, the 2D method does not highlight any issue in relation to the 1D method used for anti-doping controls, and in particular, does not show any antibody specificity issue. It is regrettable that an article creating a false impression on the 1D method based on erroneous extrapolation was published without due scientific considerations.

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Olivier Paul Rabin*
The World Anti-Doping Agency Montreal, Quebec, Canada
E-mail address: olivier.rabin@wada-ama.org.
* Corresponding author.

Francoise Lasne
Laboratoire National de Depistage du Dopage, Chatenay-Malabry, France

Jose A. Pascual
Pharmacology Research Unit, Municipal Insitute of Medical Research, Barcelona, Spain

Martial Saugy
Laboratory for Doping Analysis, Lausanne, Switzerland

Frans J. Delbeke
Peter Van Eenoo
Doping Control Laboratory, Ghent University, Ghent, Belgium

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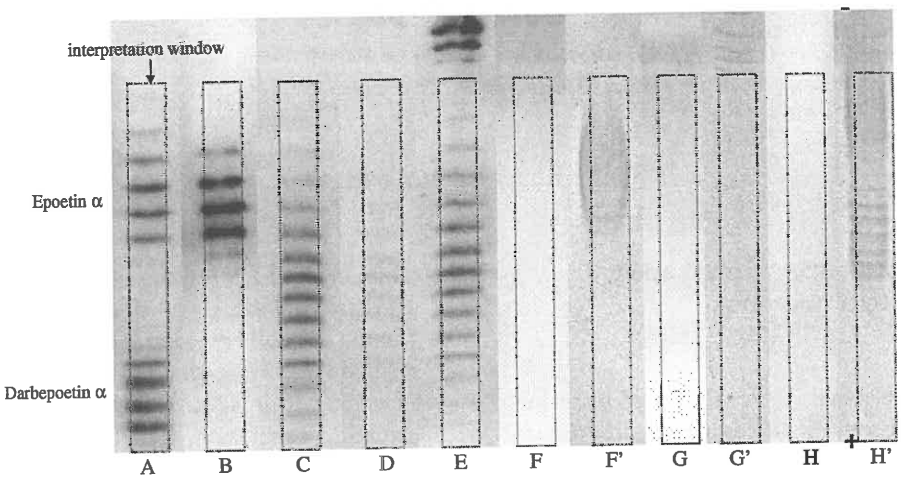

Fig. 1. Isoelectric banding patterns of EPO obtained by the 1D method used in anti-doping controls. (A) mixture of recombinant EPO Epoetin α and Darbepoetin α, (B) urine sample positive for the presence of Epoetin α (800 IU/l in the retentate), (C) and (D) urine samples negative for the presence of recombinant EPO and presenting typical patterns of natural urinary EPO (800 and 144 IU/l in the respective retentates), (E) urine sample giving rise to an additional signal unrelated to EPO outside the interpretation window, (F) α-2-HS glycoprotein precursor, (G) α-2-thiol proteinase inhibitor, (H) α-1-anti-chymotrypsin. These proteins were analysed by the 1D method at concentrations of 0.5 g/l. Their IEF patterns obtained by protein staining without immunodetection are shown in F′, G′ and H′ respectively.

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ORIGINAL PAPER

Caspase activation, sialidase release and changes in sialylation pattern of recombinant human erythropoietin produced by CHO cells in batch and fed-batch cultures

Kok Hwee Chuan · Sing Fee Lim · Laurent Martin · Chee Yong Yun · Sophia O. H. Loh · Francoise Lasne · Zhiwei Song

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Abstract The activation of caspases represents a crucial turning point during a batch or a fed-batch culture of mammalian cells. It not only affects the quantity but also the quality of the recombinant glycoprotein produced. In this study, the activation of various caspases, the release of intracellular sialidase and the changes in sialylation pattern of a recombinant product, erythropoietin (EPO), in the culture medium were analyzed in both batch and fed-batch cultures. In both setups, all caspase activities peaked at the culture time point at which decline of cell viability was most pronounced. In addition, the release of intracellular lactate dehydrogenase (LDH) was also tracked during these cultures. The increase in LDH activity in the medium coincided with the increase of intracellular caspase activities, the release of sialidase and the observed decline in cell viability, suggesting that the LDH activity in the medium can be used as an indirect indicator of apoptotic cell death in bioreactors. Isoelectric focusing (IEF) coupled with double blotting was employed to analyze the changes in sialylation pattern of the recombinant EPO. This assay resulted in a prompt resolution of secreted EPO isoforms in a time course format. IEF profile of batch culture showed relatively consistent product sialylation compared to fed-batch culture, which showed gradual band shifts towards the isoforms with fewer sialic acid as the culture progressed. These data provided a guideline for the optimal time point to terminate the culture and collect products in batch and fed-batch cultures.

Keywords Apoptosis · Caspase activation · CHO cells · Erythropoietin · Isoelectric focusing (IEF) · Lactate dehydrogenase (LDH) · Sialidase

Kok Hwee Chuan and Sing Fee Lim contributed equally to this work.

K. H. Chuan · S. F. Lim · C. Y. Yun · S. O. H. Loh · Z. Song (✉)
Bioprocessing Technology Institute,
Agency for Science, Technology and Research,
20 Biopolis Way, 06-01 Centros,
Singapore 138668, Singapore
e-mail: song_zhiwei@bti.a-star.edu.sg

L. Martin · F. Lasne
Laboratoire National de Depistage du Dopage,
143 Avenue Roger Salengro,
92290 Chatenay-Malabry, France

Introduction

Characterization of mammalian cell culture processes is essential for the economical production of therapeutic recombinant glycoproteins. The in vivo activity of many recombinant glycoproteins is highly dependent upon their glycan structures. Terminal sialic acid residues at the N-linked glycans influence a glycoprotein's circulatory

half-life significantly as it prevents the recognition by the asialo-glycoprotein receptor on the surface liver cells, which are chiefly responsible for systemic clearance of glycoproteins in the blood (Higuchi et al. 1992; Misaizu et al. 1995; Spivak and Hogans 1989; Takeuchi et al. 1989).

Desialylation of recombinant glycoproteins in cell culture media is largely attributed to the release of intracellular sialidase by dead cells (Gramer and Goochee 1993; Gramer et al. 1995). Cell death in bioreactors can be triggered by various factors. These factors include inordinate shear stress, nutrient depletion, waste accumulation, hypoxia and high osmolality (Al-Rubeai and Singh 1998; Chen et al. 2001; Ryu and Lee 1999). Under the influence of these factors, cell death in bioreactors can occur in two different forms, namely cell necrosis and apoptosis. Necrosis involves the physical rupturing of cells and is often caused by excessive mechanical agitation or aeration in bioreactors. Apoptosis, on the other hand, is a gene-regulated physiological response and is morphologically characterized by cell shrinkage, membrane blebbing, chromatin condensation and DNA fragmentation. To date, apoptosis is regarded as the principal form of cell death in cell culture (Goswami et al. 1999); though the exact pathway involved remains unclear. Breakdown of apoptotic bodies results in the release of intracellular contents, such as sialidase, into the medium which can be harmful to the recombinant product.

Apoptosis is characterized by the activation of a family of cysteine–aspartate proteases, known as caspases (Degterev et al. 2003; Hengartner 2000). In viable cells, caspases are expressed as inactive zymogens. Under the action of various apoptosis inducing signals, inactive caspase precursors are mechanistically activated. A unique hallmark of caspases is that they specifically cleave after an aspartic acid in the target protein. As different caspases prefer different amino acid sequences at the cleavage site, synthetic substrates have been generated for the analysis of various human caspases (Thornberry et al. 1997).

Apoptosis occurs through the mitochondrial pathway or the death receptor pathway (Jiang and Wang 2004; Ashkenazi and Dixit 1998). At the apex of these two apoptosis pathways are the initiator caspases, caspase-8 for the death receptor pathway and caspase-9 for the mitochondrial pathway. Active caspase-8 or caspase-9 in turn activates downstream effector caspases, such as caspase-3, caspase-6 and caspase-7, which lead to the execution of apoptosis.

Traditional methods employed in the analysis of protein glycosylation require large amount of purified product. In addition, it is a time-consuming process. As glycosylation microheterogeneity is known to occur during the course of cell culture (Floch et al. 2004; Harmon et al. 1996), alternative methods need to be developed for the routine monitoring of glycan structures of recombinant glycoproteins. Isoelectric focusing (IEF) has been successfully employed for the detection of recombinant human erythropoietin (EPO) isoforms in urine samples of athletes (Lasne and de Ceaurriz 2000; Lasne et al. 2002). Separation of EPO glycoforms is based on pI differences due to different sialylation profiles exhibited by recombinant and endogenous EPO. In contrast to traditional glycan analysis approaches, IEF requires a much smaller sample size and it eliminates the need for complicated protein purification and sample preparation. It does not compromise the resolution as IEF can clearly distinguish one isoform from another that differs by only one sialic acid residue.

Though IEF was previously employed in the analysis of EPO glycoforms in batch culture (Yoon et al. 2003, 2004), the published method required reverse phase HPLC purified EPO samples of up to 10 μg. In this paper, we proposed the use of isoelectric focusing, in conjunction with double blotting (Lasne 2001, 2003), as a simplified method to analyze the sialylation profile of secreted EPO produced by a recombinant CHO cell line in both batch and fed-batch cultures, without the need for any form of preliminary sample purification. Due to the sensitivity and specificity of double blotting, sample requirement was reduced by 50-fold to only 200 ng.

In addition to sialidase, many other cellular components could be released by the dead cells. One such component is lactate dehydrogenase (LDH). As LDH can be easily monitored we used LDH as an indicator of cell death during a batch and a fed-batch culture. When both the

productivity and the quality (sialylation pattern) of the recombinant EPO in the medium were considered, our data provided a guideline as when to stop the bioreactor and collect the products.

Materials and methods

Cell line and culture conditions

CHO-K1 EPO HT # 1 was a single clone recombinant CHO cell line that stably expressed recombinant human erythropoietin with a six His tag attached to the C-terminal. It was engineered by transfecting a CHO-K1 (ATCC CRL-9618) cell line with a pcDNA 3.1(+) (Invitrogen) construct, containing human erythropoietin cDNA tagged with six histidine codons at the 3′ end. Stable transfectants were selected using G418 (Gibco) at 1 mg ml^{-1} and single clones were isolated and maintained in DMEM (Gibco) supplemented with 10% (v/v) FBS (Gibco).

CHO-K1 EPO HT # 1 was adapted to serum free suspension culture and maintained in HyQ PF-CHO (Hyclone), supplemented with 2 g l^{-1} sodium bicarbonate (Sigma) and 0.1% (v/v) Pluronic F-68 (Gibco). Glucose and glutamine concentrations were 17 and 4 mM, respectively. Suspension culture was agitated via an orbital shaker (Ika) set at 110 rpm and kept in a 37°C humidified incubator with 8% (v/v) CO_2.

Bioreactor setup and set point controls

Two-liter doubled-walled, round-bottom glass vessels with heated water jackets (B. Braun Biotech) were employed in the bioreactor runs. Exponentially growing cells from a 2nd day seed culture were used to inoculate both the batch and the fed-batch bioreactors at a seeding density of 3.0×10^5 cells ml^{-1}. The initial working volume of each bioreactor was 1.5 l. Process control set points were controlled using a digital control unit (DCU, B. Braun Biotech). Each bioreactor was configured with a standard 60 mm diameter, 45° pitch blade impeller and agitation rate was set at 110 rpm. Aeration was achieved through the headspace and silicone membrane tubing basket (B. Braun Biotech). Dissolved oxygen (DO) concentration was monitored using a polarographic electrode (Mettler Toledo) and maintained at 50% air saturation at 1 atm using an air/N_2 mix or O_2/air mix set at 1 slpm. Culture pH was controlled at 7.1, using intermittent CO_2 gas sparging (maximum 0.2 slpm) or 7.5% (w/v) $NaHCO_3$ solution. Culture temperature was controlled at 37°C via the integrated water jacket. HyQ PF-CHO was employed as the initial basal medium for both batch and fed-batch cultures.

Feeding strategy for the fed-batch operation

Two separate feed media were formulated for controlling glucose and glutamine concentrations. The glutamine feed medium was prepared from a proprietary blend comprising of 10 × salt-free, glucose-free, and glutamine-free DMEM/F12 (Hyclone), supplemented with 1 × HyQ PF-CHO. Glutamine concentration was 200 mM (Sigma). The glucose feed medium was a 1 M glucose solution (Sigma).

Predictive feed forward control was employed in the fed-batch setup. Discrete control of glutamine and glucose were achieved by feeding the projected amounts of glutamine and glucose that would be consumed by the cells over the forecast intervals. This was estimated by assuming that the specific growth rate, specific glucose and glutamine consumption rates during the forecast interval would be the same as that in the current sampling interval (Sauer et al. 2000). Glucose and glutamine concentration set points were 0.5 and 0.15 g l^{-1}, respectively. Periodically, about 10 ml of culture were sampled from each reactor twice a day at 9 AM and 5 PM. Culture supernatants and cell pellets, after centrifugation at 2,000 rpm (Beckman), were aliquoted for various analyses and kept at −20°C.

Cell density and cell viability determination

Viable cell density and cell viability of culture samples were measured using Cedex (Innovatis), based on trypan blue staining. Standard deviation obtained in each reading (based on 20 slide images) was used in the computation of cell count error.

EPO quantification

EPO concentration of supernatant samples was determined using an enzyme-linked immunosorbent assay (ELISA) kit (Roche). Appropriate serial dilutions were performed on samples prior to ELISA analysis. ELISA assay was duplicated for each sample to ensure reproducibility.

Extracellular sialidase activity analysis

Extracellular sialidase activity was determined by fluorescence emission according to a modified method (Gramer and Goochee 1993), using 4-methylumbelliferyl-α-D-*N*-acetylneuraminic acid (4MU-NANA, Sigma) as substrate. Eleven µl of 4-MU-NANA stock solution (3.3 mM 4-MU-NANA in 0.9 M KH_2PO_4 pH 7.2) was added to 89 µl of supernatant sample and the reaction mixture was incubated at 37°C. The fluorescence generated was measured at intervals of 2 min for 2 h using a microplate reader (Tecan GENios) at an excitation wavelength of 365 nm and emission wavelength of 450 nm. Extracellular sialidase activity of each sample was calculated based on the rate of increase in fluorescence. Samples were run in duplicate to ensure reproducibility.

Lactate dehydrogenase (LDH) activity analysis

LDH activity in the culture media was analyzed using a Cytotoxic Detection Kit (Roche) according to the supplied manufacturer's protocol. Medium samples from batch culture were used as undiluted while samples from fed-batch cultures were diluted 10 × with PBS. Samples were run in duplicate to ensure reproducibility.

Intracellular caspase activities assay

Harvested CHO cell pellets were lysed with chilled lysis buffer (5 mM DTT, 10 mM HEPES pH 7.5, 2 mM EDTA, 0.1% CHAPS) on ice for 10 min and cell lysates were collected via centrifugation at 13,000 rpm for 5 min (Eppendorf). Protein concentration of these cell lysates was assayed using Coomassie Plus (Pierce) against known BSA protein standards (Pierce) by determining absorbance at 595 nm. Samples containing equal loading of total protein were incubated with their respective assay buffers and para-nitroaniline (pNA) labeled caspase substrates in excess at 37°C in 96 well plates. Release of free pNA chromophores from cleaved substrates by caspases were monitored by tracking changes in absorbance at wavelength of 405 nm, using a microplate reader (Tecan) over a period of 2.5 h in 15 min intervals. Caspase activity of each sample was subsequently calculated from the rate of increase in free pNA. Caspase substrates used were DEVD-pNA (for caspase-3), IETD-pNA (for caspase-8), LEHD-pNA (for caspase-9), VDVAD-pNA (for caspase-2), WEHD-pNA (for caspase-1, –4 and –5) and VEID-pNA (for caspase-6). Buffers and substrates were used as instructed in Chemicon's colorimetric assay kits (APT163, APT167, APT169, APT173, APT165 and APT171) for the respective caspases.

Western blotting assay for caspase-3 activation

Harvested CHO cell pellets were lysed and protein concentration of these cell lysates was assayed using methods described in the previous section. Cell lysates containing 50 µg of protein were loaded for each sample, resolved via 12% polyacrylamide SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membrane (Bio-rad). Rabbit polyclonal antibody which recognizes the full length human caspase-3 and the large fragment of caspase-3 resulting from cleavage (Cell Signaling) was used as primary antibody, while horseradish-peroxidase (HRP) conjugated goat anti-rabbit IgG (H + L) antibody (Jackson ImmunoResearch) was employed as secondary antibody. Detection was carried out using ECL detection reagent (Amersham Biosciences) and exposed using chemiluminescent detection film (Roche).

Isoelectric focusing (IEF)

Supernatant samples were concentrated via Microcon® (Millipore) or diluted to 1000 IU EPO/L with dilution buffer (1% BSA, 50 mM Tris–HCl pH 7.4) prior to isoelectric focusing in a

1.3 mm thick, 5% T, 3% C polyacrylamide gel (Bio-rad) containing 7 M urea (Fluka), 1.2% (w/v) 2–4, 1.2% (w/v) 4–6, 1.2% (w/v) 6–8, 0.4 % (w/v) 2–11 ampholytes (Serva) and 5% (w/v) sucrose (Sigma). After prefocusing at 250 V and 10°C for 30 min (using 0.5 M NaOH as catholyte and 0.5 M H_3PO_4 as anolyte), 20 µl of diluted/concentrated samples were applied onto rectangular pieces of filter paper, placed 0.5 cm from the cathode end of the gel. Electrophoresis was conducted on the Multiphor II Electrophoresis system (Amersham-Pharmacia) at 1 W per cm of gel length and stopped at 3,600 Vh. EPO isoforms were specifically revealed by a double-blotting method using monoclonal anti-human EPO AE7A5 (R&D Systems) as primary antibody and biotinylated goat anti-mouse IgG (H + L) (Pierce) as secondary antibody, according to previously described methods (Lasne 2001, 2003). Chemiluminescent images of these IEF blots were detected with a charge coupled device (CCD) camera (Fuji), using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce).

Luminescence intensity analysis and sialylation quality evaluation

Luminescence intensity spectrum corresponding to the isoelectric profile of each sample was obtained using "AIDA 1D-Evaluation" software from Fuji. Quantitative sialylation quality of each sample was evaluated based on both the distribution of luminescence intensity and the degree of sialylation associated with the respective isoforms.

Results

Growth profiles of batch and fed-batch cultures

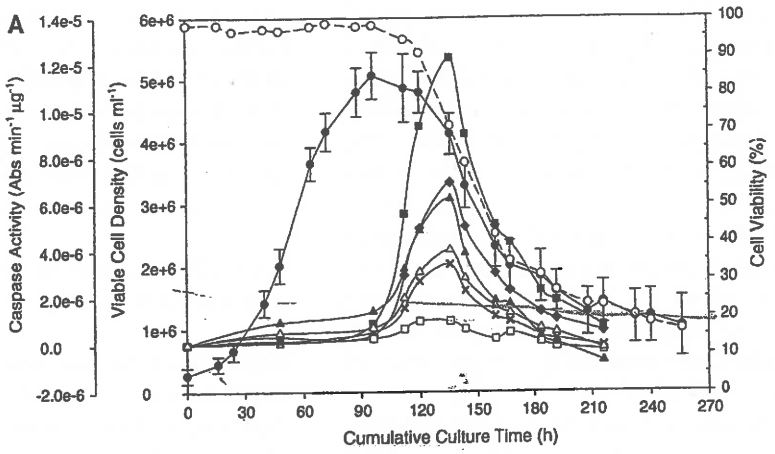
Viable cell density profiles of both batch and fed-batch cultures of CHO-K1 EPO HT #1 exhibited a trend as shown in Fig. 1A, B, with characteristic logarithmic, stationary and death phases. A maximum viable cell density of 5.07×10^6 cell ml^{-1} was observed at a culture time of 96 h for batch culture. Maximum viable cell density of fed-batch culture reached 8.56×10^6 cell ml^{-1} at a culture time of 136 h. Cumulative integral viable cell density showed an approximate 2-fold increase in fed-batch culture over batch culture. Decline in culture viabilities was observed at 120 and 168 h for batch and fed-batch cultures, respectively.

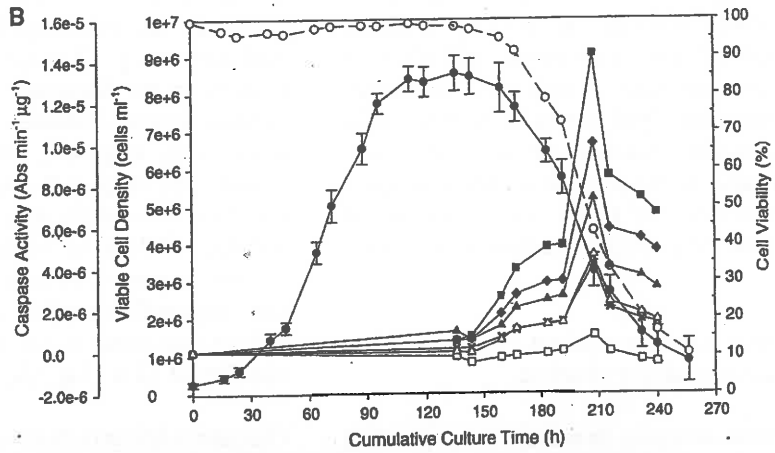
Intracellular caspase activity profiles of batch and fed-batch cultures

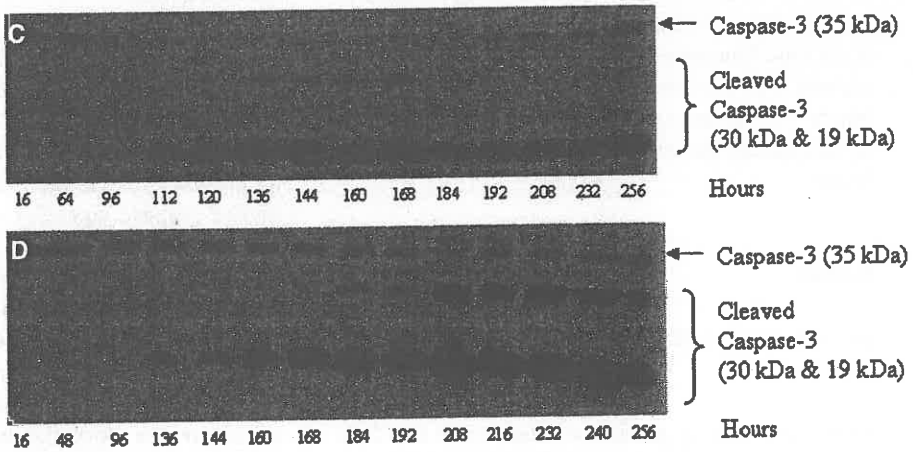
Intracellular caspase activity profiles during the entire course of batch and fed-batch cultures, in a time course format, were previously unreported. The intracellular caspase activities at various time points, in both batch and fed-batch cultures, were analyzed using Chemicon's caspase assay kits, containing six different substrates designed for various human caspases. As shown in Fig. 1A, intracellular caspase-2, caspase-3, caspase-6, caspase-8 and caspase-9 like activity peaks were observed at a culture time point of 136 h and cell viability of 71% in batch culture. In fed-batch culture, caspase-2, caspase-3, caspase-6, caspase-8 and caspase-9 like activity peaks were located at a culture time point of 208 h and corresponding cell viability of 43% (Fig. 1B).

Caspase-3 Western blots of batch and fed-batch cultures

Activation of inactive zymogene of caspases is often used as a benchmark to signify the occurrence of apoptotic death. Among the 14 identified caspases, cleavage of effector caspase-3 is often employed by investigators to verify the activation of the caspase signaling cascade (Li et al. 2001; Tse and Rabbitts 2000). Western blot was performed to track the cleavage of caspase-3 in a time course format for batch and fed-batch cultures. As shown in Fig. 1C, D, inactive caspase-3 zymogene (35 kDa) was cleaved into smaller fragments (30 and 19 kDa) during the later stages of culture in both batch and fed-batch setups, which further accentuated that apoptosis was indeed activated in batch and fed-batch cultures. The 19 kDa fragment is the large subunit of active capsase-3. In batch culture, cleavage of capase-3 was first observed at 112 h of culture,







◄**Fig. 1** Cell viability and caspase activation during batch and fed-batch cultures. (**A**) Viable cell density, cell viability and intracellular caspase-2, –3, –5, –6, –8, –9 like activity profiles of batch culture. (•) Viable cell density; (○) cell viability; (♦) caspase-2 like activity; (■) caspase-3 like activity; (□) caspase-5 like activity; (▲) caspase-6 like activity; (×) caspase-8 like activity; (△) caspase-9 like activity. (**B**) Viable cell density, cell viability and intracellular caspase-2, –3, –5, –6, –8, –9 like activity profiles of fed-batch culture. (**C**) Western blot analysis of caspase-3 activation during the course of a batch culture. (**D**) Western blot analysis of caspase-3 activation during the course of a fed-batch culture

which corresponded to a culture viability of 94.2%. Similarly, in fed-batch culture, cleavage of capase-3 was first observed at 136 h of culture, which corresponded to a culture viability of 98.1%. Maximum amounts of cleaved caspase-3 subunits were detected at 136 and 208 h in batch and fed-batch cultures, respectively, which concurred with the occurrence of the caspase-3 activity peaks in the caspase activity assay. This antibody was known to recognize human, mouse and rat caspase-3, as shown here, it also recognize Chinese hamster caspase-3. Since the antibody only recognizes the full length and the large fragment (p20 subunit) of caspase-3, the small subunit of caspase-3 was not detected.

Extracellular sialidase activity profiles of batch and fed-batch cultures

The release of sialidase from dead cells into culture medium and the concomitant desialylation of recombinant product is a major problem caused by apoptosis during cell culture processes. The sialidase activities of batch and fed-batch culture media at various time points are shown in Fig. 2A, B. The sialidase activities of both batch and fed-batch cultures, during the initial 120 h of culture were found to be consistently low, which implied that sialidase release was not a secretory event. Peak sialidase activities of both batch and fed-batch cultures, occurred at a sample time point subsequent of the caspase-3 activity peak. Coupled with the fact that the initial increase in sialidase activities for both batch and fed-batch cultures (see arrows in Fig. 2A, B) were accompanied by a marked increase in caspase activities; these observations suggest that extracellular sialidase activities, in both batch and fed-batch cultures, were due to intracellular sialidase release, which in turn was an event downstream of apoptosis. The sialidase activity in the fed-batch culture medium was much higher than that in the batch culture medium could be due to the higher cell density in the fed-batch culture.

LDH activity profiles of batch and fed-batch cultures

The release of LDH has been used as a generic indicator of cell death (Wagner et al. 1992). LDH activity in the culture medium of both batch and fed-batch cultures were tested and the results are shown in Fig. 2A, B. The initial increase in LDH activities in both batch and fed-batch medium coincided with the increase in sialidase activity in the media and the increase in caspase activities. The onset of cell death occurred at the same time point for both methods (indicated by arrows in Fig. 2A, B). Intuitively, LDH release could also indicate that significant amount of other intracellular enzymes, such as proteases and glycosidases, were also indiscriminately released into the medium during the death phase of culture.

Volumetric EPO yield in batch and fed-batch cultures

In batch culture, a peak EPO concentration of 0.9 mg l^{-1} was obtained at a culture time point of 112 h, which was 32 h before the observed caspase-3 peak (Fig. 3A). The reason for this premature halt in EPO production could be due to nutrient depletion, which was inevitable in batch culture. In fed-batch culture, a peak EPO concentration of 4.61 mg l^{-1} was established at a culture time point of 208 h. This was the same time point at which caspase activities peaked (Fig. 3B). In fed-batch culture, volumetric EPO yield continued to escalate even at rather low cell viabilities (70 → 45%) as shown in Fig. 3B. This could be due to the availability of nutrients in fed-batch culture, which provided an impetus for the remaining viable cells to actively secrete EPO. Once these cells are committed towards apoptosis (signified by the observed caspase-3 peak), these cells would cease to produce the recombinant product.

IEF profiles of EPO in batch and fed-batch cultures

The sialylation quality of secreted EPO at various culture time points in both batch and fed-batch cultures was analyzed by IEF. Sialylation quality was evaluated based on the sialylation pattern of secreted EPO resolved through IEF. As shown in Fig. 4A, a highly consistent EPO sialylation profile was observed throughout the entire batch culture. The most sialylated EPO product, the band I, which was the nearest to the anode, was not detected after a culture period of 184 h. In the fed-batch culture, gradual band shifts could be observed from Fig. 4B as the culture progressed. The most sialylated EPO product, the band I, was not detected after 144 h, while bands, II and III, which were progressively less sialylated, were not detected in the medium after 192 and 216 h, respectively.

In batch culture, sialidase activity peaked at a culture time point of 184 h, concurring with the loss of the most sialylated band I in the IEF profile (Figs. 2A, 4A). The peak sialidase activity of fed-batch culture occurred at a culture time of 216 h and corresponded with the loss of band III in the IEF profile (Figs. 2B, 4B). Loss of bands I, II and III in fed-batch culture and loss of band I in batch culture occurred at normalized sialidase activities of 0.29, 1.12, 2.03 and 0.64 FU min^{-1} μl^{-1} of media, respectively. Significant loss of hyper-sialylated species in fed-batch culture during the death phase, as compared to batch culture, could be attributed to a larger total cell density, which directly correlated to larger amount of sialidase released, leading to more extensive desialylation.

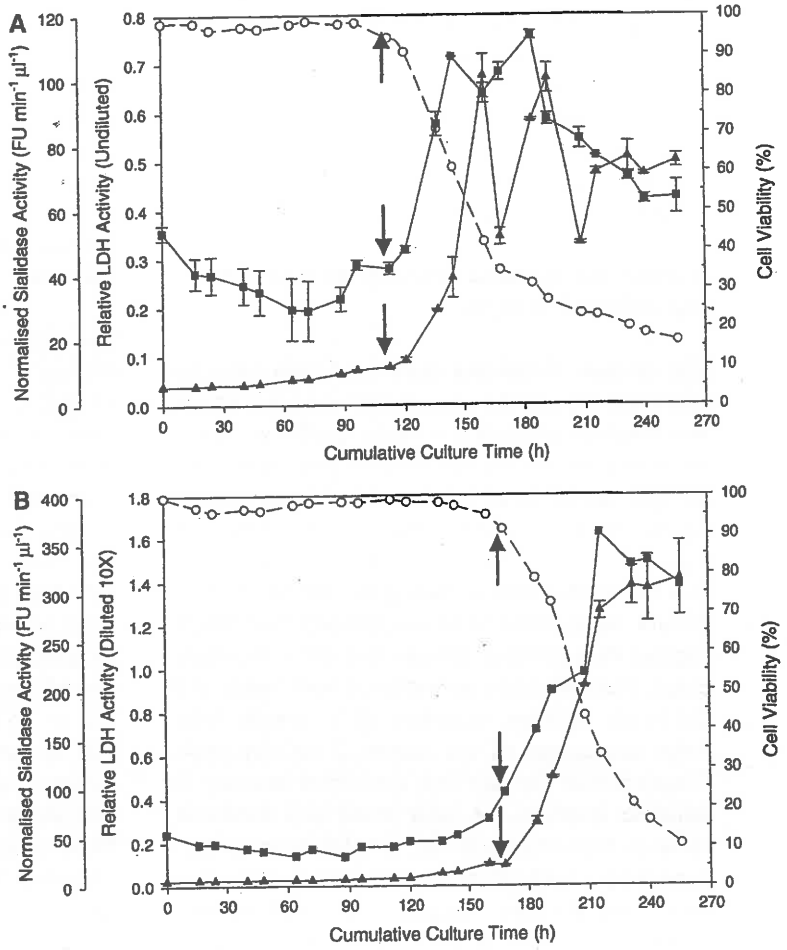


Fig. 2 Release of cellular sialidase and LDH in batch and fed-batch cultures. (A) Cell viability, extracellular sialidase activity and LDH activity profiles of the same batch culture shown in Fig. 1A. (○) Cell viability; (▲) LDH activity; (■) sialidase activity. (B) Cell viability, extracellular sialidase activity and LDH activity profiles of the same fed-batch culture shown in Fig. 1B

A band intensity spectrum corresponding to an early batch culture sample is shown in Fig. 4C. Peaks to the right correspond to the more sialylated EPO isoforms, while percentages displayed on top of peaks represent the relative distribution of each EPO isoform in the resolved culture sample. A quantitative analysis of these band intensity spectrums is shown in Fig. 5. These data represent the relative total sialic acid on the EPO molecule at different time points. In contrast to batch culture, sialylation quality of EPO in fed-batch culture deteriorated even more during the late logarithmic to stationary phase of culture. It should be pointed out that the derived numerical index shown in Fig. 5 is not an absolute quantification of EPO sialic acid content but a relative comparison between product sialylation of EPO at various time points of both batch and fed-batch cultures.

Discussion

Apoptosis is broadly regarded as the principal mechanism responsible for death in cell cultures (Goswami et al. 1999; Vives et al. 2003). Our results further confirmed this proposition. In both batch and fed-batch cultures, all caspase activities peaked at the same time point at which cell viability declined most abruptly. In spite of the fact that caspase-8 and caspase-9 are initiator caspases, both enzymes peaked at the same time point as effector caspase-3 and caspase-6. This could be attributed to the large interval between consecutive samplings (8 or 16 h), which limited the sensitivity of this assay (since enzymatic reactions involving initiator and effector caspase activation are likely to be rather spontaneous). When

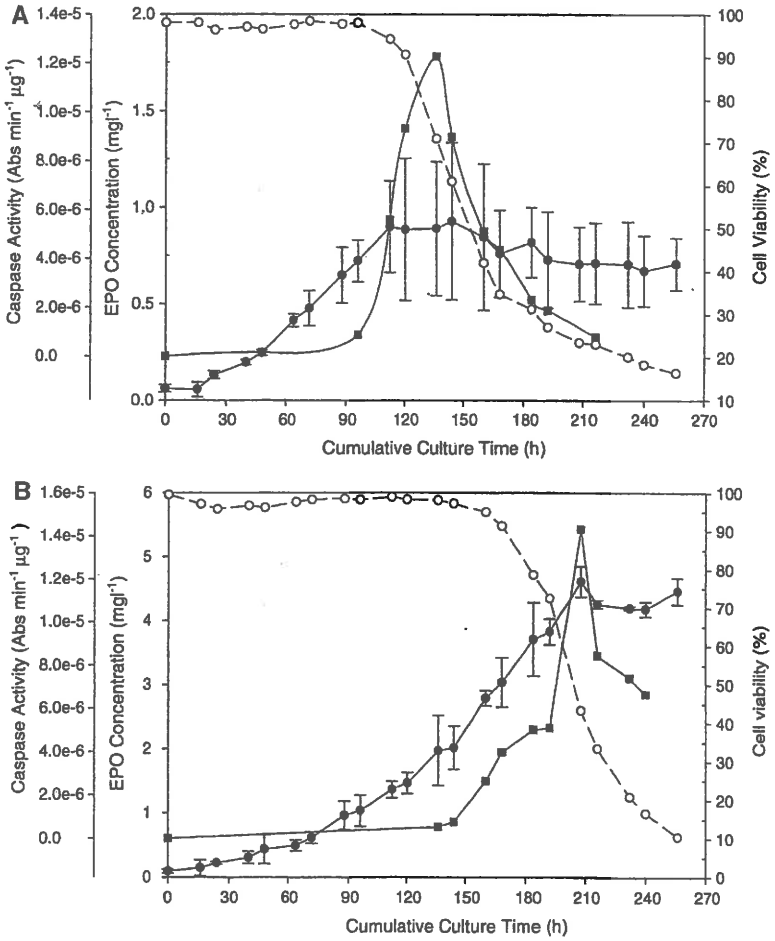


Fig. 3 Productivity of EPO in batch and fed-batch cultures. (**A**) Volumetric EPO yield, intracellular caspase-3 like activity and cell viability profiles of the same batch culture shown in Fig. 1A. (•) EPO concentration; (■) caspase-3 like activity; (O) cell viability. (**B**) Volumetric EPO yield, intracellular caspase-3 like activity and cell viability profiles of the same fed-batch culture shown in Fig. 1B. (•) EPO concentration; (■) caspase-3 like activity; (O) cell viability

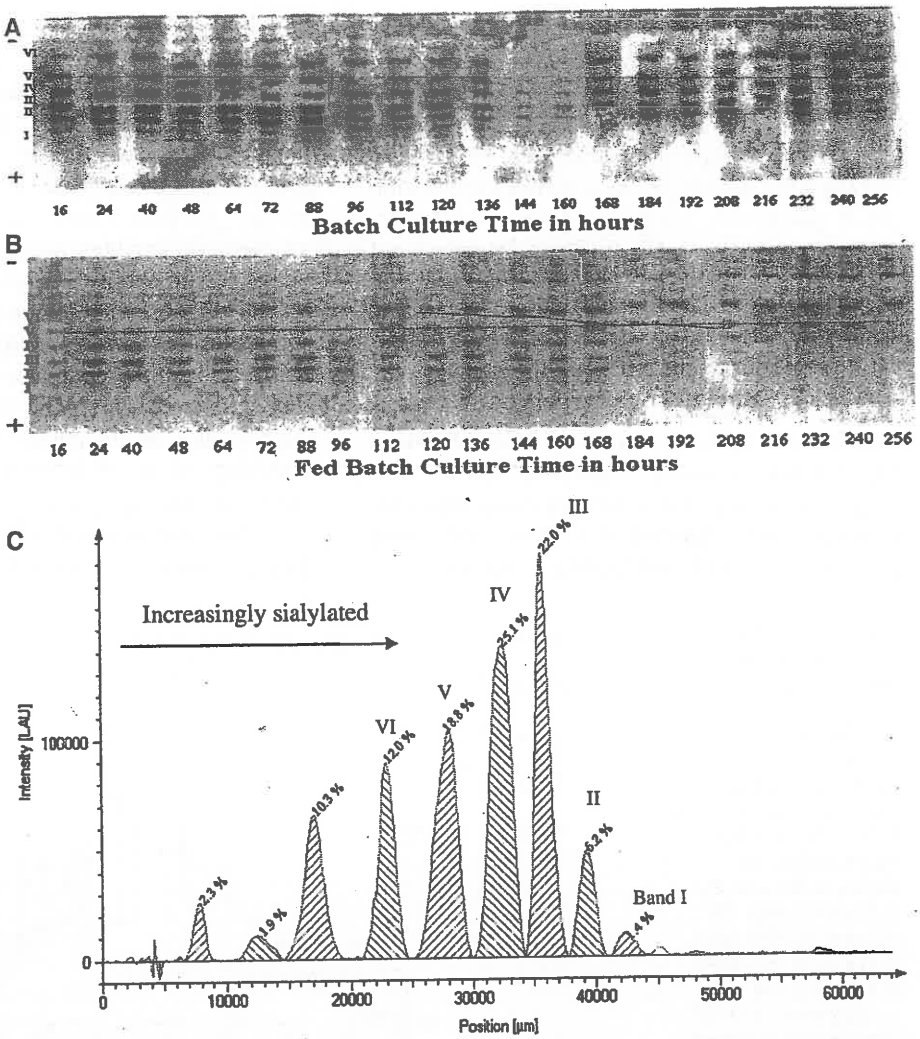


Fig. 4 Changes in sialylation pattern of EPO in batch and fed-batch cultures analyzed with IEF. (**A**) IEF profile of secreted EPO in the batch culture. (**B**) IEF profile of secreted EPO in the fed-batch culture. (**C**) Band intensity spectrum of a batch culture sample obtained using "AIDA 1D-Evaluation" software from Fuji

analyzed with these synthetic short peptides as substrates, peak activity of caspase-3 was the highest, followed by caspase-2, caspase-6, caspase-9 and caspase-8.

Caspase-1, –4 and –5 activities were absent in both batch and fed-batch cultures since the substrate (WEHD-pNA) for these three caspases was not cleaved. This observation was not surprising. Caspase-1 was reported to be involved specifically in inflammation rather than apoptosis (Zeuner et al. 1999), and thus unlikely to be activated under batch and fed-batch culture conditions. Caspase-4 and –5 were not found in the mouse genome (Reed et al. 2003) and have not been reported in rats either. It is very likely that caspase-4 and –5 would be absent in CHO cells, since Chinese hamsters are closely related to rats and mice.

The apoptotic pathways involved in batch and fed-batch cultures could be different, since the fundamental aim of fed-batch culture is to overcome nutrient limitation in batch culture. However, results from the caspase activity assay seem to imply that both caspase-8 and caspase-9 were

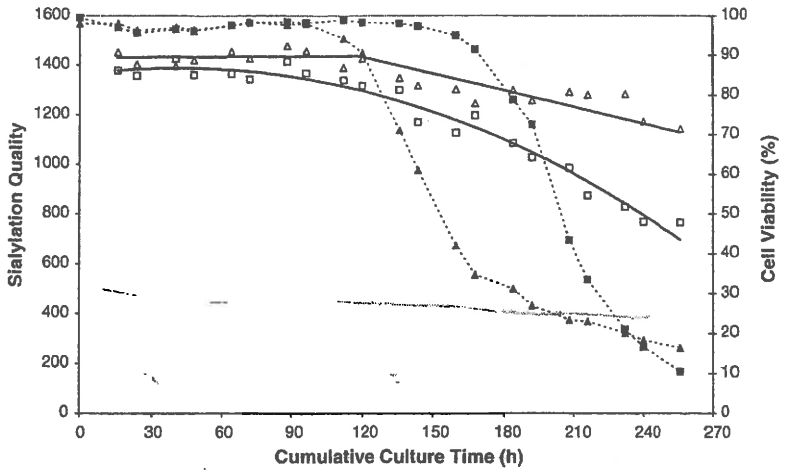


Fig. 5 Sialylation quality profile of EPO in batch and fed-batch cultures and its association with cell viability. (△) Sialylation quality score of the batch culture; (□) sialylation quality score of the fed-batch culture; (▲) cell viability of the batch culture; (■) cell viability of the fed-batch culture. The numbers shown here is not an absolute quantification of the sialic acid content of EPO but a relative comparison of sialylation between EPO products at various time points of both batch and fed-batch cultures

activated in batch and fed-batch cultures, suggesting that both the mitochondrial and death receptor pathways might be involved. However, before any conclusion can be reached, there is a need to demonstrate the actual activation of the respective caspases in batch or fed-batch cultures, using alternative methods such as immuno-blotting. Although a particular substrate may be cleaved most efficiently by a specific caspase, many other caspases can also contribute to the cleavage of that substrate, possibly with a lower efficiency. For example, the cleavage of "caspase-8 substrate" shown in Fig. 1A, B should be treated as a combined effect of several caspases, i.e. caspase-8 itself and possibly several other caspases. According to Thornberry et al. (1997), these caspase substrates were derived combinatorially, based on specificity for recombinant human caspases; but even then, these sequences were demonstrated not to be highly specific in their caspase recognition abilities (Talanian et al. 1997).

Intracellular sialidase release has been reported to be the chief determinant for the deterioration in sialylation profiles of recombinant cell cultures during the death phase (Gramer and Goochee 1993; Gramer et al. 1995). The loss of the three most sialylated EPO bands and the appearance of several less sialylated bands during the death phase of fed-batch culture (Fig. 4B) closely correlated with the increase in extracellular sialidase activity, suggesting sialic acid residues on the EPO molecules could possibly be liberated by sialidase action. However, extent of sialylation of EPO in fed-batch culture was observed to be inferior to that of batch culture (Fig. 5), even during the late logarithmic phase of culture. This observation was inferred from the disappearance of the most sialylated band I, in fed-batch culture after only 144 h. Since sialidase activity detected in the medium during this time frame was found to be low (~0.29 FU min^{-1} μl^{-1}), this phenomenon is unlikely to be caused by sialidase release.

After a careful analysis of the data shown in Figs. 2B, 5, we realized that the sialylation of EPO in the fed-batch culture started to drop even when the sialidase activity in the medium was still relatively low. A possible explanation for this observation is that the sialylation of recombinant EPO was affected by other factors. Presence of ammonium in culture medium has been reported to reduce both the extent of sialylation and antennarity of glycan structures (Yang and Butler 2000a, 2000b, 2002). In these experiments, the ammonium concentration was rather high in the

fed-batch culture during the late logarithmic to stationary phase as compared to that of the batch culture (data not shown). Influx of less sialylated EPO variants secreted into the medium during this time frame is likely to adulterate and/or inundate hypersialylated EPO produced during the early logarithmic phase when ammonium concentration was low. This explanation would also justify the relatively "basic" sialylation profile that was detected by IEF during late logarithmic to stationary phase of culture, in addition to the action of the sialidase in the medium.

Lactate dehydrogenase (LDH) assay is a cell death assay based on measurement of LDH activity in the culture medium. LDH is a cytosolic enzyme but is rapidly released into the medium upon loss of membrane integrity. This assay was originally used to measure cell death via necrosis (Koh and Choi 1987). Lately it has been successfully used to measure apoptotic cell death as well (Lobner 2000; Koh et al. 1995; Koh and Cotman 1992). We clearly demonstrated that in both batch and fed-batch cultures the increase of LDH in the medium coincided with the activation of all the caspases inside the cell. Therefore, the release of LDH into the medium is very likely due to apoptotic death, rather than necrotic death. Thus, the increase of LDH activity in the culture medium can be used as an indicator for apoptotic cell death in the bioreactors.

In conclusion, we have systematically analyzed the activation of various caspases and the release of sialidase and LDH into the medium during batch and fed-batch cultures. All the caspase activities peaked at the time points when the viability dropped most dramatically. At the same time, sialidase and LDH, possibly other cellular contents as well, were released into the medium. As a result, the recombinant product, EPO in this case, gradually lost its sialic acid. IEF can be employed to monitor this change throughout the entire course of the culture. The key parameters, such as product yield and extent of product sialylation, can be used to optimize the harvest time point. Since LDH in the medium can be easily monitored it can serve as an indirect marker for apoptotic cell death in bioreactors.

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To the editor:

No doubt about the validity of the urine test for detection of recombinant human erythropoietin

Beullens et al report the "false-positive detection of recombinant human erythropoietin in urine following strenuous physical exercise."[1(p4711)] This report, based on observations conducted on urine from 1 single subject, relies in fact on serious errors of interpretation of poor-quality images. A first sample, collected just after exercise and analyzed by double-blotting following isoelectric focusing of the retentate from ultrafiltrated urine,[2,3] gives rise to a banding pattern interpreted as unrelated to Epo based on the argument that this pattern is missing in a second sample collected 1 hour later. A simple routine assay (using other antibodies than the AE7A5 used for immunoblot) of the Epo level in these 2 ultrafiltered samples before IEF would have probably shown that a high concentration of this hormone was present in the first one but not in the second one. It is quite surprising that this basic control

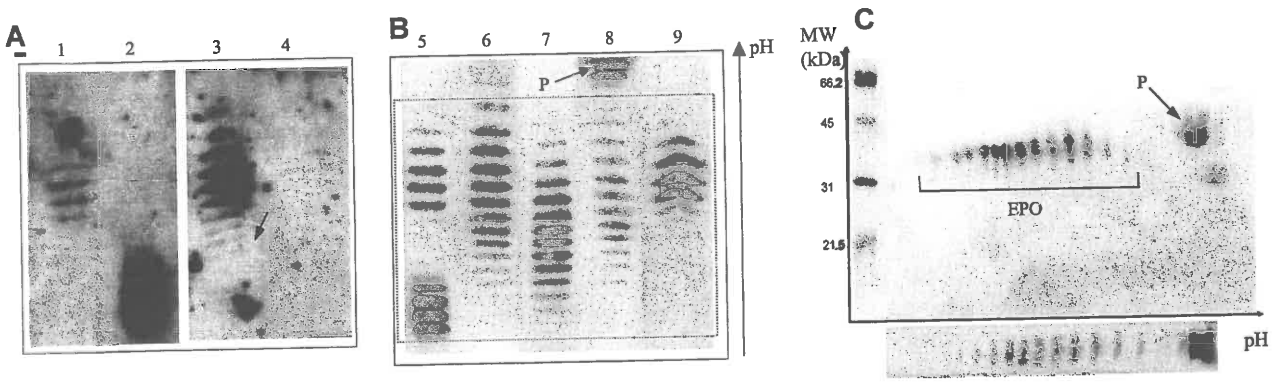

Figure 1. Epo profiles obtained by double-blotting. Panel A is reproduced from Beullens et al.[1(Fig1A)], and panels B and C are results from our laboratory. (A) Lane 1 shows epoetin β; lane 2, darbepoetin α; lane 3, urine sample considered as "false positive" (the arrow shows a white hole corresponding to ineffective transfer of proteins in this area); and lane 4, the same sample as in lane 3 1 hour later. (B) Lane 5 shows a mixture of epoetin β and darbepoetin α; lane 6, natural urinary Epo from a sample taken after strenuous exercise (note some shift toward the cathode of the banding pattern); lane 7, natural urinary Epo; lane 8, urine sample showing the protein (P) unrelated to Epo outside the window of integration (dotted box) used for interpretation of an antidoping control result; and lane 9, urine sample in case of epoetin administration (note the difference with the natural urinary Epo pattern even in the case of the post–strenuous exercise sample). (C) Two-dimensional electrophoresis of a urine sample showing both Epo and protein P.

was not performed. Did the authors fail to obtain an image of natural urinary Epo for comparison with their "interfering protein"? The SDS electrophoresis results are entirely misinterpreted due to the very different conditions chosen for preparation of the same urine sample before SDS (10-fold concentration) and IEF (200-fold concentration). Under such distorted conditions, the absence of a band corresponding to the molecular weight of Epo (39 kDa) in SDS electrophoresis cannot, in any account, be considered as a proof that there was no EPO in the sample submitted to IEF. The only band detected (42 kDa) by SDS has been incorrectly related to the bands detected by IEF. Why isn't a 2-dimensional electrophoresis shown to demonstrate such an assertion? In fact, the bands shown in the IEF figure cannot be detected in the SDS experiment due to the insufficient concentrating step. From our experience, the 42-kDa band corresponds to a protein very often present in urine in high concentrations after strenuous exercise and detected by the AE7A5 antibody used for immunoblotting. This protein is known not to interfere with the Epo pattern of an antidoping control due to a more basic isoelectric point (pI). It is unfortunate that the IEF image shown in this article has been cut just below the area corresponding to this protein. The subsequent investigations of deglycosylation were very interesting and corroborate our results about this 42-kDa protein. It is unfortunate that they were arbitrarily attributed to the bands shown by IEF.

In summary, the IEF pattern shown by Beullens et al has been interpreted as unrelated to Epo, whereas it corresponded to a bad-quality image (such a result would have been categorically rejected from any interpretation in antidoping control) of a well-known Epo pattern observed after particular conditions of strenuous exercise. The figure enables one to compare the IEF results of Beullens et al[1] with well-identified Epo patterns as observed in our laboratory from several hundreds of samples. A 2-dimensional electrophoresis has been introduced to support our statements.

It is disappointing that such poor-quality experiments and misinterpreted results led the authors to believe that the validity of the Epo antidoping test could be questioned.

Françoise Lasne

Correspondence: Laboratoire National de Dépistage du Dopage, 143, avenue Roger Salengro, Châtenay-Malabry, 92290, France; e-mail: f.lasne@lndd.com.

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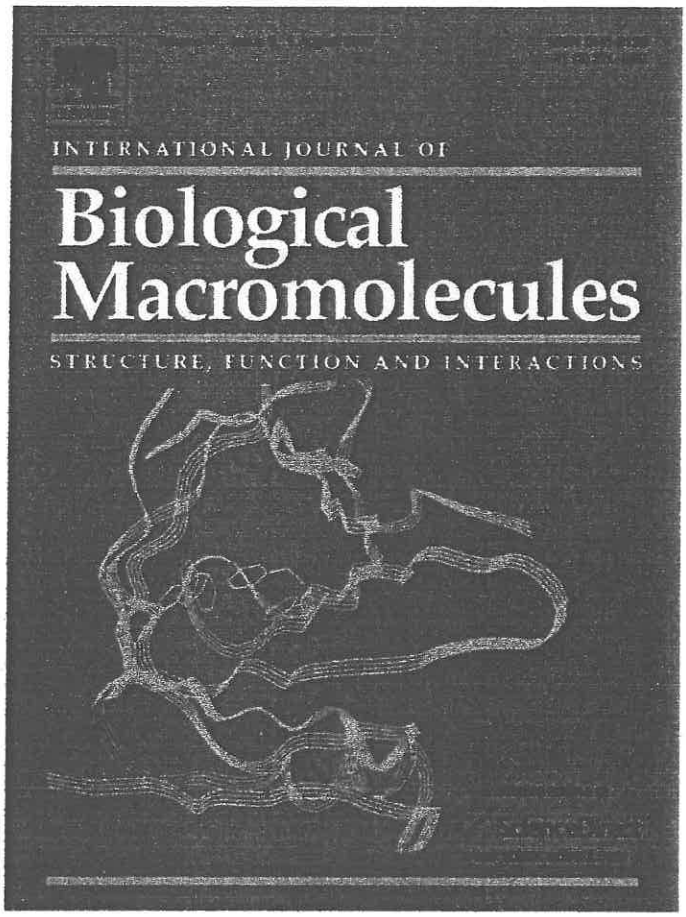

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Short communication

Isoelectric profiles of human erythropoietin are different in serum and urine

Françoise Lasne*, Laurent Martin, Jean Antoine Martin, Jacques de Ceaurriz

Agence Française de Lutte contre le Dopage, Département des Analyses, 143, Avenue Roger Salengro, 92290 Châtenay-Malabry, France

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Abstract

By adding a step of immunoaffinity to the method we had previously developed for analysing erythropoietin (EPO) in urine, we were able to study the isoelectric profiles of this hormone in human serum samples. This method was sensitive enough to investigate samples presenting physiological levels of this hormone. Comparison with the corresponding profiles in urine showed that natural EPO was systematically more acidic in urine. The acidification process, which was not patent in the non-human primate *Cynomolgus* macaque, clearly also affected recombinant EPO when injected into humans. This process was unrelated to any enzymatic activity in urine since the incubation of natural or recombinant EPO in urine induced no transformation of their isoelectric profiles. The nature and mechanism of the structural modifications occurring during the renal handling of this hormone remain to be investigated.

Keywords: Erythropoietin; Isoelectric profiles; Serum/urine comparison

1. Introduction

Human erythropoietin (EPO), a glycoproteic hormone mainly produced by kidney cells, has generated a great deal of interest from scientists, the pharmaceutical industry and, unfortunately, athletes intent on doping. Its stimulating effects on erythropoiesis have been well documented and are the basis for its use as a drug [1]. Additional biological effects, such as neuroprotective activity, have been discovered and are under investigation [2]. This hormone was initially purified by Miyake in 1977 from the urine of patients with aplastic anemia [3]. After its gene was cloned [4,5], recombinant human erythropoietin (rHuEPO) became available and was initially used to treat the anemia induced by renal failure [6]. Since that time, the indications for rHuEPO have expanded [7]. The entire structural characterization of EPO has been performed on either purified natural urinary hormone [8,9] or, for the most part, the corresponding recombinant protein [9,10]. Some differences in the glycosylation of natural urinary and rHuEPO have been described [11] and the differences in their respective isoelectric profiles provide the basis for anti-doping control at the present time [12]. The sparse data concerning serum EPO, on the other hand, have been obtained from a small number of anemic patients presenting abnormally high circulating levels of this hormone [13,14]. This can be explained by the technical difficulties related to the very low concentration of EPO (normal range: about 30–170 ng/L) in a very high protein content (60–80 g/L) in serum. We developed a procedure to study the isoelectric profile of natural serum EPO and compare it with the corresponding profile in urine under physiological conditions. In addition, this method made it possible to compare the serum and urine EPO profiles after administration of recombinant hormone. These comparisons raise interesting questions for future investigations.

2. Method

Thirty-five pairs of serum and urine samples were obtained from 35 healthy control subjects and an additional 7 pairs were obtained from rHuEPO-treated volunteers during a trial of Epoetin alfa administration [15] (Eprex from Janssen-Cilag, sub-cutaneous injections, 260 IU/kg; protocol approved by the Ethics Committee of Montpellier Hospital, France). In addition,

Abbreviations: EPO, erythropoietin; rHuEPO, recombinant human erythropoietin

* Corresponding author. Tel.: +33 1 46 60 28 69; fax: +33 1 46 60 30 17.
E-mail address: f.lasne@afld.fr (F. Lasne).

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one urine sample was obtained from a patient with renal failure who was treated by Epoetin beta (Recormon from Roche, intra-venous injections 217 IU/kg, three times).

For comparison, serum and urine samples from Cynomolgus macaques involved in a trial of EPO cDNA transfer into skeletal muscle [16] (protocol approved by the Institutional Animal Care and Use Committee of the University of Nantes, France) were analyzed before any gene transfer.

All samples were kept at −20 °C until analysis.

The isoelectric profiles of urinary EPO were obtained as previously described [17]. Briefly, 18 mL of urine were concentrated by ultra-filtration (about 500 times) using membranes with a molecular weight cut-off of 30,000 Da and the retentate was submitted to isoelectric focusing in a pH gradient 2–6. EPO was specifically detected using the double-blotting method [18] with monoclonal anti-human EPO AE7A5 from R&D Systems. This provided a chemiluminescent image of the EPO isoforms.

Due to the very high protein content in serum, a preliminary step to extract EPO by immunoaffinity was necessary before ultra-filtration: monoclonal anti-human EPO antibodies (clone 9C21D11 from R&D Systems) were coupled to Affi-Gel Hz hydrazide gel from Bio-Rad and columns were prepared, stored and conditioned according to the manufacturer's instructions. Each of the samples (4 mL) was applied four times consecutively. After the column was washed with 0.01 M phosphate buffer, pH 7.4, containing 0.637 M NaCl, EPO was eluted with 0.4 M glycine NaOH, pH 11, 6 M urea, 0.01% Tween 20 and 0.025% bovine serum albumin. The eluates were then submitted to ultra-filtration, IEF and double-blotting as described for the urine samples [17]. This procedure could be used for urine samples. In this case, the immunoaffinity step was introduced after the initial ultrafiltration step.

3. Results and discussion

Before the interpretation of any result obtained using immunoaffinity, this step was tested to ensure that it did not induce an artefactual modification of the real isoelectric pattern of EPO. This was confirmed by the complete recovery of unmodified isoelectric profiles for the Biological Reference Preparation (from the European Pharmacopeia Commission), Darbepoetin alfa (from Amgen) and the Second International Biological Standard of human urinary erythropoietin (from NIBSC) after the immunoaffinity step (Fig. 1). The relative intensities of the different bands composing a pattern after immunoaffinity were systematically inside the 95% confidence interval estimated from 11 patterns of the same sample without immunoaffinity. As an illustration, Table 1 provides the values corresponding to the Second International Biological Standard of human urinary erythropoietin shown in Fig. 1. The choice of the antibody and the elution conditions were essential to ensure both the integrity of the profile and a recovery compatible with physiological serum levels of EPO.

In addition to its main purpose, which was to study serum EPO, the immunoaffinity step provided an opportunity to definitively put to rest criticisms concerning the specificity of the test used for anti-doping control in urine [19]. As expected,

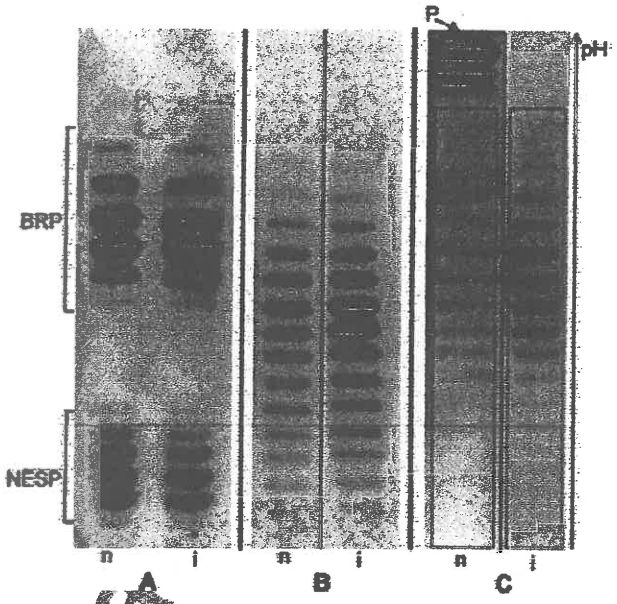

Fig. 1. Non-selectivity of the immunoaffinity step toward the different isoforms of EPO: qualitative results. Chemiluminescent images showing the isoelectric profiles of: (A) biological reference preparation (BRP) (equimolecular mixture of recombinant Epoetins alfa and beta) and Darbepoetin alfa (NESP); (B) Second International Biological Standard of human urinary erythropoietin. All were identical whether they had been submitted to the additional step of immunoaffinity (i) or not (n). (C) For urine samples, the only modification induced by immunoaffinity was the disappearance of the previously described protein P (appearing in some urine samples) outside the window of integration (dotted box). Inside this window, which corresponded to the EPO isoforms, the bands were similar, whether the urine samples had been submitted to the additional step of immunoaffinity (i) or not (n). Due to the concentrating effect of immunoaffinity, the bands are clearly more intense in (i). The anode is at the bottom of the figure.

the additional specificity of the 9C21D11 antibody used for immunoaffinity made only the isoforms of the protein previously described as unrelated to EPO [20] disappear. This protein, being situated outside the window of integration used for interpretation of a profile, does not interfere with the result of an anti-doping control. The essential demonstration was that inside the window, the same isoforms were detected, whether the urine sample was submitted to the additional step of immunoaffinity or not. The only difference was the intensification of the EPO bands by the concentrating effect of immunoaffinity (Fig. 1).

As shown in Fig. 2, the isoelectric patterns of serum EPO appeared to be highly heterogeneous, being composed of more than 10 isoforms in a mean *pI* range of 4.1–4.9 that is more basic than the *pI* range of 3.8–4.7 previously described for urine EPO [12]. Though the serum profiles presented some differences between individuals, their most intense isoforms were systematically more basic than in the corresponding urine profiles and a roughly similar acidic shift was observed from serum to urine in individuals. Interestingly, this acidification was not evident in the Cynomolgus macaques, which presented much more similar EPO patterns in serum and urine.

In the humans injected with recombinant EPO, the pattern of the drug was recovered in serum without any modification. A

Table 1
Non-selectivity of the immunoaffinity step toward the different isoforms of EPO, quantitative results

Band number	1	2	3	4	5
RI (%) without IA, mean	0.9	2.1	5	10.1	13.4
RI (%) without IA, CI	0–1.9	1.3–2.9	3.1–6.9	7.7–12.4	9.8–17.1
RI (%) with IA	0.4	2.1	4.8	10.1	13.2
Band number	6	7	8	9	10
RI (%) without IA, mean	14.5	13.6	11.3	10.2	7.9
RI (%) without IA, CI	12.8–16.4	11–16.3	9.3–13.1	8.7–12.7	6.1–9.7
RI (%) with IA	16	15.3	11.4	8.4	7.3
Band number	11	12	13	[illegible]	15
RI (%) without IA, mean	5	3.6	1.7	[illegible]	0.2
RI (%) without IA, CI	3.8–6.2	0.5–6.6	0.9–2.6	0.2–[illegible]	0–0.4
RI (%) with IA	5.2	3.2	1.9	[illegible]	0

Relative intensities (RI) of the different bands composing the pattern of the Second International Biological Standard of human urinary erythropoietin shown in Fig. 1. The bands were numbered from the cathode to the anode. For each of the bands, the RI observed after immunoaffinity (IA) may be compared to the mean and the 95% confidence interval (CI) calculated from 11 repeated analysis without IA of the same sample.

slight but very clear acidification of this pattern was systematically observed in the corresponding urine samples. Interestingly, one exception to this rule was observed in the case of the patient with chronic renal failure who was treated with recombinant EPO. In this case, the EPO pattern recovered in urine was strictly identical to the pattern of the injected drug, indicating that the acidification process was ineffective.

The mechanism of the acidification process is unknown. It does not seem related to an enzymatic activity of urine since incubation in urine of EPO immunoextracted from serum or pure rHuEPO did not give rise to any change in their respective isoelectric profiles (data not shown). The acidification that occurs during the renal processing of EPO cannot be explained by the glomerular charge selectivity (negative charge barrier) which would be expected to select the most basic isoforms of human serum hormone for excretion in urine.

During the practice of anti-doping control, it has been noted that after some strenuous exercises, the urinary pattern, though unequivocally interpreted as natural EPO, is slightly shifted toward more basic pI. In view of our results in serum, it may be envisaged that the temporary proteinuria (including a significant rise in the CHO level) occurring after exercise exceeds the capacity of the mechanisms involved in the acidification of EPO and thus gives rise to more basic isoforms in urine.

In the same manner, the very high proteinuria of the patient with renal failure (6.3 g/24 h) might be the indirect reason for the defective acidification of the administered recombinant hormone. However, a direct failure in the acidification function may also have occurred.

Given the differences between the human serum and urine EPO isoelectric profiles, it is clear that the structural data obtained from urinary EPO must be considered as specific for the urinary species of this hormone. These observations deserve further investigation to identify the structural differences between serum and urine hormones and to explore the mechanisms involved in this transformation.

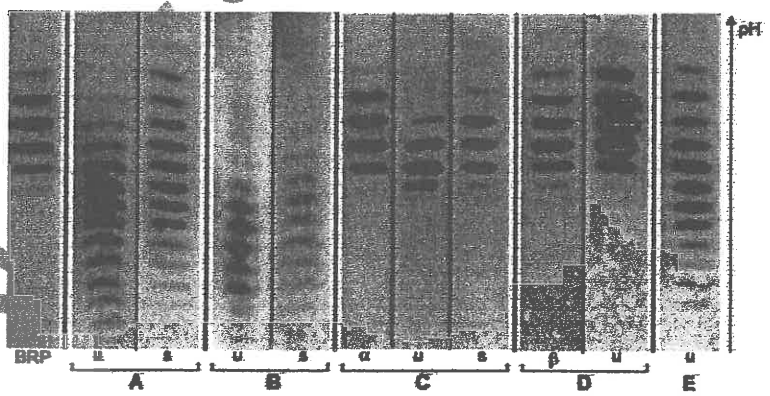

Fig. 2. Comparison of serum and urine isoelectric profiles of EPO. Chemiluminescent images of (A) natural endogenous EPO profiles from human urine (u) and the corresponding serum (s). Note the acidification of the profile in urine. (B) Natural endogenous EPO profiles from cynomolgus macaque urine (u) and the corresponding serum (s). Note the similarity of the profiles in serum and urine. (C) Recombinant EPO (Epoetin alfa) profiles from the pharmaceutical preparation (α), urine (u) and the corresponding serum (s) from healthy human volunteers injected with this drug (samples obtained 48 h after sub-cutaneous injection of 260 IU/kg). Note the acidification of the profile in urine. (D) Recombinant EPO (Epoetin beta) profile from the pharmaceutical preparation (β) and urine (u) from a patient with chronic renal failure injected with this drug. No acidification of the injected drug appeared in the urine. (E) Natural endogenous EPO profile as observed in some urine samples after strenuous physical exercises. The anode is at the bottom of the figure.

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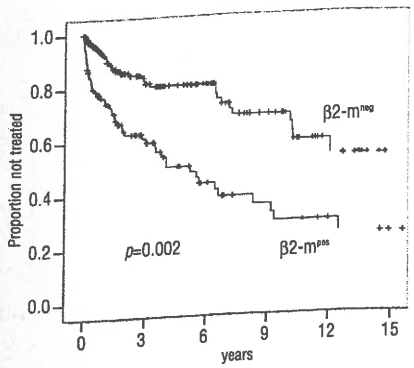
A



B

Risk categories	Univariate analysis HR (95% C.I., *p* value)
No factor	1
One factor	1,5 (0.7-3.4, *p*=ns)
Two factor	5.0 (2.5-10.2, *p*<0.0001)
Three factors	15.4 (7.3-32.5, *p*<0.0001)

C

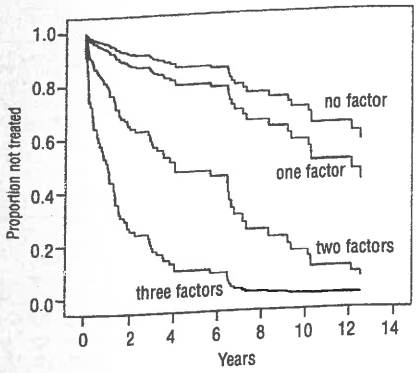


Figure 1. (A) Kaplan–Meier estimates of treatment-free survival TFS. Comparison between β2-m^{neg} patients versus β2-m^{pos} patients. (B) Cox univariate analysis for the predictive value of marker combinations for the risk categories. (C) Cox derived estimated TTT curves according to the combination of the three prognostic factors.

ly increase together with the progressive expansion of the leukemic clone suggesting a close correlation between stage which measures tumor burden and β2-m levels. Although a correlation with disease stage likely exists, there was a substantial proportion of patients with high β2-m levels already at Binet A stage (low tumor burden). Possibly, CLL cells from these patients are more activated *in vivo* and shed more abundant β2-m. Taken all the above into consideration, the data indicate that the role of β2-m as a prognostic tool should be re-evaluated possibly in prospective studies involving large patient cohorts.

Massimo Gentile,[1] Giovanna Cutrona,[2] Antonino Neri,[3] Stefano Molica,[4] Manlio Ferrarini,[2,5] and Fortunato Morabito[1]

[1]Unità Operativa Complessa di Ematologia, Azienda Ospedaliera di Cosenza, Cosenza; [2]Divisione di Oncologia Medica C, Istituto Nazionale per la Ricerca sul Cancro, IST, Genova; [3]Centro per lo Studio delle Leucemie, Dipartimento di Scienze Mediche, Università di Milano, Unità Operativa di Ematologia 1, Fondazione IRCCS Policlinico, Milano; [4]Dipartimento di Oncologia/Ematologia, Azienda Ospedaliera Pugliese-Ciaccio, Catanzaro; [5]Dipartimento di Oncologia, Biologia e Genetica Università degli Studi di Genova, Italy.

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Key words: β2-microglobulin, CD38, IgVH mutational status, chronic lymphocytic leukemia, prognosis.

Correspondence: Fortunato Morabito, Unità Operativa Complessa di Ematologia, Dipartimento di Medicina Interna, Azienda Ospedaliera di Cosenza, Viale della Repubblica, 87100 Cosenza, Italy. Phone: international +39.0984.681329. Fax: international +39.0984.791751. E-mail: fortunato_morabito@tin.it

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Detection of continuous erythropoietin receptor activator in blood and urine in anti-doping control

Anti-doping control of erythropoietin Epo relies on the differentiation by isoelectric profile of natural endogenous hormone from the recombinant hormone used for doping. The first and second generations of recombinant Epo were detectable in urine.[1,2] The third generation, Continuous Erythropoietin Receptor Activator CERA, was obtained by linking a methoxy polyethylene glycol to epoetin β, a first generation rHuEPO, resulting in significantly greater stability in blood. CERA, approved in Europe in July 2007, was expected to be quickly misused in sport. Several questions were thus raised about its detectability. We demon-

strate here that detection in blood poses no problem and that partial excretion in urine occurs after some physical exercise. Our analytical results from the Tour de France 2008 anti-doping control, which tested both urine and blood samples, serve as an illustration.

Staining revealed that the isoelectric pattern of pure CERA was composed of seven bands with apparent isoelectric points in the pI 4.8-5.2 range Figure 1. This was surprising since CERA that results from pegylation of epoetin β via the amino group of lysine residues was expected to be more acidic than epoetin β. One explanation could be a shielding effect of the PEG shell on some surface charges of epoetin. In any case, the isoelectric pattern clearly differentiated CERA from natural Epo.

However, analysis of Epo isoelectric patterns in urine or plasma as previously described[2,3] required two monoclonal anti-human Epo antibodies. Briefly, 18 mL of urine or 1 mL of plasma were first submitted to a preparation step including immunoaffinity chromatography using clone 9C21D11 and ultrafiltration at a 30-kDa molecular weight cut-off. The retentates from ultrafiltration were then submitted to isoelectric focusing and Epo was detected by double-blotting, using clone AE7A5.[4,5] The final result was a chemiluminescent image of the isoelectric patterns of Epo.

Due to the polymer chain in the molecule, the CERA structure might not be recognized by the antibodies used in this method. In fact, use of AE7A5 only (double-blotting only) and both AE7A5 and 9C21D11 (immunochromatography and double-blotting) provided patterns identical to that obtained without antibody stained gel Figure 1.Thus, although the amino terminus of the polypeptidic chain is one of three major pegylation sites, the epitope of AE7A5, which is within the first 26 amino acids, was still accessible to this antibody and the epitope of the 9C21D11 antibody not identified was also not affected by pegylation. The sensitivity of the method was determined to be less than 40 pg applied onto the IEF gel (corresponding to initial 50 pg/mL in 1 mL serum and 3 pg/mL in 18 mL urine).

These results proved that, technically, the usual method for Epo analysis could detect CERA. However, due to its hydrodynamic volume that is critical for glomerular filtration, CERA detection in urine, the main biological medium for anti-doping control, seemed unlikely.

Anti-doping control of recombinant Epo during the Tour de France 2008 was performed as usual from urine. CERA was clearly detected in two urine samples (both from the same athlete) reported as positive. Other samples from this same athlete and 3 others gave rise to faint CERA images and were considered highly suspicious. Blood samples taken before the race and mid-race were retrospectively analyzed. All eight plasma samples two per athlete from the 4 athletes having positive or suspicious urine samples gave rise to very clear CERA images. This confirmed that only a small part of CERA was excreted from blood into urine. However, strenuous exercise enhances protein excretion in urine by increasing glomerular permeability and decreasing tubular reabsorption.[6-8] CERA excretion in urine is affected by exercise as well. This is illustrated by the case of the athlete reported positive from two urine samples. Two positive blood samples were taken 11 days apart while

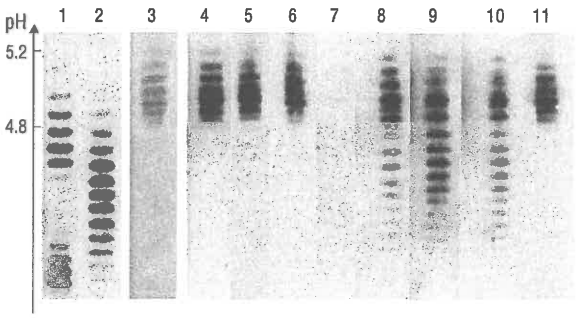

Figure 1. Isoelectric patterns of Epo. All the patterns are chemiluminescent images except in lane 3. Lane 1, mixture of Epoetin beta, a first generation rHuEpo up, and Darbepoetin alfa, a second generation rHuEpo down; lane 2, natural urinary Epo; lane 3, pure CERA Coomassie Blue stained gel; lane 4, pure CERA analyzed by double-blotting only; lane 5, CERA added to a urine sample and analyzed by immunochromatography and double-blotting. Note that in addition to the characteristic pattern of CERA, some less intense bands corresponding to the endogenous Epo of this sample were detected in a more acidic area. The next lanes correspond to samples from an athlete of the Tour de France 2008 (see Figure 2 for details of chronology): lane 6, first plasma sample presence of CERA; lane 7, first urine sample (no Epo detected, neither natural nor CERA); lane 8, second urine sample (presence of CERA); lane 9, third urine sample traces of CERA; lane 10, fourth urine sample presence of CERA; lane 11, second plasma sample presence of CERA. Note the presence of additional isoforms to those of CERA in lanes 8 to 10, corresponding to the natural endogenous Epo in urine. This latter is not visible in plasma samples (lanes 6, 11) due to the very intense signal of CERA, which is present in a much greater proportion in plasma than in urine.

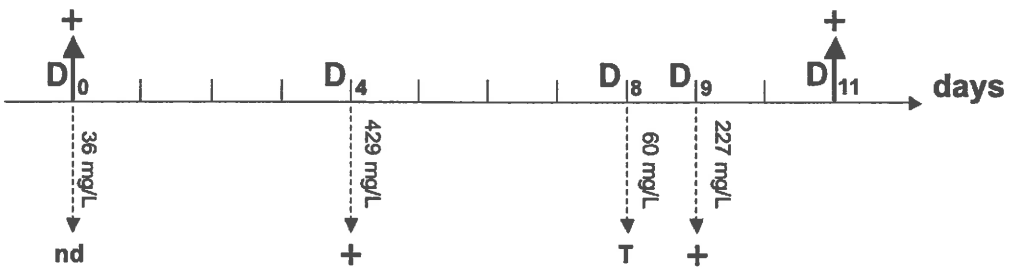

Figure 2. Chronology of sampling in the athlete reported positive for CERA from urine samples during the Tour de France 2008. The days of sampling are indicated by up and down arrows for blood and urine, respectively. The ends of the arrows point to the results of the analyses: +, positive; T, traces; nd, no detectable Epo neither natural nor CERA. D 0 corresponds to the day before the start of the race. Along the down arrows are indicated the protein levels of the urine samples. The isoelectric patterns of Epo obtained from these samples are shown in Figure 1.

Effects of Exercise on the Isoelectric Patterns of Erythropoietin

Séverine Lamon, MSc, Laurent Martin, BSc,† Neil Robinson, PhD,* Martial Saugy, PhD,* Jacques de Ceaurriz, PhD,† and Françoise Lasne, MD†*

Objectives: Recombinant erythropoietin has a strong impact on aerobic power and is therefore one of the most potent doping agents in endurance sports. The anti-doping control of this synthetic hormone relies on the detection, in the urine, of its isoelectric pattern, which differs from that of the corresponding natural hormone, the latter being typically more acidic than the former. However, a small number of natural urinary patterns, referred to as "atypical patterns," are less acidic than the dominant form. Based on anecdotal evidence, the occurrence of such patterns seems to be related to particular strenuous exercises. This study aimed to demonstrate this relation using a strenuous exercise protocol.

Design: Seven athletes took part in a training protocol including a series of supramaximal short-duration exercises. Urine and blood samples were collected throughout the protocols.

Settings: World Cycling Center, Aigle, Switzerland, and research laboratories.

Participants: Seven top-level athletes (cyclists) were involved in this study.

Main Outcome Measures: Erythropoietin (EPO) isoelectric patterns were obtained by submitting blood and urine samples to isoelectric focusing. Additional protein dosages were performed.

Results: Supramaximal short-duration exercises induced the transformation of typical urinary natural EPO patterns into atypical ones. None of the obtained atypical patterns fulfilled the 3 criteria mandatory for reporting an adverse analytical finding. Serum EPO patterns were not affected by the exercises that caused the transformation of urinary patterns.

Conclusion: An exercise-induced transient renal dysfunction is proposed as a hypothetic explanation for these observations that rely on parallel investigations of proteinuria in the same samples.

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From the *Laboratoire Suisse d'Analyse du Dopage, Centre Universitaire Romand de Médecine Légale, Centre Hospitalier Universitaire Vaudois, University of Lausanne, Epalinges, Switzerland; and †Département des Analyses, Agence Française de Lutte contre le Dopage, Châtenay-Malabry, France.

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Reprints: Séverine Lamon, MSc, Laboratoire Suisse d'Analyse du Dopage, Centre Universitaire Romand de Médecine Légale, Ch des Croisettes 22, 1066 Epalinges, Switzerland (e-mail: severine.lamon@chuv.ch).

Key Words: effort, erythropoietin, doping, urine

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INTRODUCTION

Erythropoietin (EPO) is a 30.4-kDa human glycoprotein hormone produced primarily by the kidney[1] and to a small extent by the liver.[2] Its production is stimulated by tissue hypoxia, and its main physiological effect is the stimulation of erythropoiesis.[3] By increasing the number of circulating erythrocytes, EPO improves the oxygen-carrying capacity of blood, leading to an increased oxygenation of tissues.[4]

Because of these biological effects, EPO acts as a potent enhancer of athletic performance in endurance sports. Unsurprisingly, human recombinant erythropoietin (rHuEPO) has been used as a doping agent to improve aerobic power[5] ever since it has become available in 1987. In 1990, the International Olympic Committee Medical Commission banned the misuse of this drug, even though rHuEPO detection was not possible at that time. It is only in 2000 that an rHuEPO detection method became available as part of anti-doping control.[6] This method relies on the differentiation of isoelectric patterns generated by natural endogenous and recombinant forms of the hormone that are excreted in the urine. The difference in the isoelectric patterns owes to the fact that the most acidic isoforms of natural EPO are absent in the recombinant hormone because of nonidentical posttranslational modifications.

However, important variabilities in isoelectric patterns can also be observed for natural EPO when different urine samples are compared. Although most samples yield typical acidic patterns, other patterns, referred to as "atypical," present a shift of their isoforms toward a more basic isoelectric point (pI). Such atypical natural patterns are taken into account in anti-doping control procedures: To demonstrate the presence of rHuEPO,[7] a strict distribution of the basic isoforms of the hormone must be present. Based on the observations of Paris and Lausanne anti-doping laboratories, atypical patterns seem to occur in urine samples collected after strenuous exercise. Accordingly, such samples are commonly referred to as "effort urines."

The objective of this study was to positively demonstrate that atypical patterns can indeed be induced in urine by strenuous effort. To this aim, an exercise protocol was set up with top-level athletes performing exercises on a cycling track in a controlled manner. The evolution of urinary EPO patterns

was followed throughout the protocol. In addition, serum EPO and urinary proteins were also measured in an attempt to understand how such atypical patterns were generated.

MATERIALS AND METHODS

Subjects

All the investigations were conducted according to the Declaration of Helsinki as amended by the 41st World Medical Assembly (Hong Kong 1989) and were approved by the local ethical committee. All participants gave their informed consent and agreed to blood tests and urine collection. The training protocol included 7 top-level cyclists, 5 men and 2 women aged between 18 and 26 years, and took place in the frame of the World Cycling Center (Aigle, Switzerland).

Training Protocol

Before the strenuous exercises, which took place in the morning, the 7 subjects performed an appropriate 60-minute warm-up, followed by 15 minutes of active recovery and 15 minutes of complete rest. After that, each male athlete had to perform an intensive exercise composed of four 500-m rides on the cycling track (rolling start) at maximum speed, separated by 25 minutes of active recovery. One female athlete had to perform three 500-m rides and the other one to perform three 600-m rides, separated by 25 minutes of active recovery. The same training session was repeated in the afternoon by the second female athlete only (subject 2).

Sample Collection

Ideally, 4 urine samples were collected at different times for all subjects: the first morning urine (a), 1 urine sample collected before the warm-up training (b), 1 urine sample collected in the middle of the effort (c), and 1 urine sample collected directly at the end of the effort (d). Two hours separated the collection of the a-urine and the b-urine, whereas about 3 hours separated the collection of the b-urine and the d-urine. In the case of the female athlete who performed a second training session (subject 2), 2 additional urine samples were collected: the first one 3 hours after the end of the morning session (e) and the second one at the end of the afternoon session (f). These 2 last sample collections were separated by 3 hours.

At each collection time, the athletes were asked to completely empty their bladder. The samples were immediately aliquoted and frozen at −20°C until analysis.

In addition, 2 blood samples were collected using Serum Gel monovettes (Sarstedt, Numbrecht, Germany): the first one before (b) and the second one after the training session (d). Blood samples were immediately centrifuged. The resulting serum samples were then aliquoted and frozen at −80°C until analysis.

Total Protein, EPO, and Retinol-Binding Protein Quantification

Total protein content in urine samples was measured using a Coomassie Blue Plus Protein assay reagent kit (Pierce, Rockford, Illinois). Erythropoietin levels were determined in the retentates obtained from ultrafiltration of urine using enzyme-linked immunosorbent assay (ELISA) (human EPO Quantikine IVD; R&D Systems, Inc, Minneapolis, Minnesota). To estimate EPO levels in the original urine samples, the results obtained by ELISA were divided by the concentration factor of ultrafiltration. Retinol-binding protein (RBP) levels in urine were directly determined by ELISA (RBP4 ELISA kit; Immundiagnostik AG, Bensheim, Germany). In all cases, the results were corrected according to the specific gravity of the urine samples assayed.

Analysis of EPO Isoelectric Patterns

Erythropoietin isoelectric patterns were determined as previously described by Lasne et al[6] with an additional preliminary step to extract EPO by immunoaffinity,[8] as summarized below. Although this step was absolutely required only for serum samples, because of their very high protein content, it was also applied to the urine samples.

The samples were first subjected to an immunoaffinity step using monoclonal anti-human EPO antibodies (clone 9C21D11 from R&D Systems) coupled to Affi-Gel Hz hydrazide gel (Bio Rad). This step was followed by ultrafiltration through membranes with a molecular weight cutoff of 30,000 Da to concentrate the hormone in the retentate. The retentates were then submitted to isoelectric focusing in a pH gradient of 2 to 6, and the EPO isoforms were specifically revealed by the double-blotting method,[9] using monoclonal anti-human EPO antibodies (clone AE7A5 from R&D Systems). Chemiluminescent imaging of the EPO isoforms was carried out with an LAS-1000 plus CCD camera (Fuji). Advance image data analyzer 1D (v. 3.44) software was used to analyze the images and generate corresponding isoelectric profiles. A reference sample, consisting of an equimolecular mixture of recombinant epoetins α and β [the Biological Reference Preparation (BRP) from the European Pharmacopoeia Commission], was systematically included in the different isoelectric focusing runs as a position marker. The bands obtained with the BRP were numbered from 0 to 5 (counting from the anode to the cathode). The relative intensities of the different isoforms were evaluated for each sample using a fixed densitometer window (5 mm width) that included all the bands of any given lane. Baselines of the different lanes were drawn valley to valley for each peak in a profile, and the relative intensity was calculated as the percentage of the peak integral reduced by the background intensity in comparison with the sum of all the peaks of the profile. A "basic area" was defined from the cathode edge of the window up to and including band 1 of BRP (Figure 1). The percentage of basic isoforms (PBI) was calculated as the sum of the relative intensities of the isoforms situated in the basic area.

RESULTS

EPO Isoelectric Patterns Prior, During, and After the Effort

A clear shift of the urinary EPO isoforms toward a more basic pI was induced by exercise in all 7 subjects. The evolution of the EPO pattern from the first morning urine to

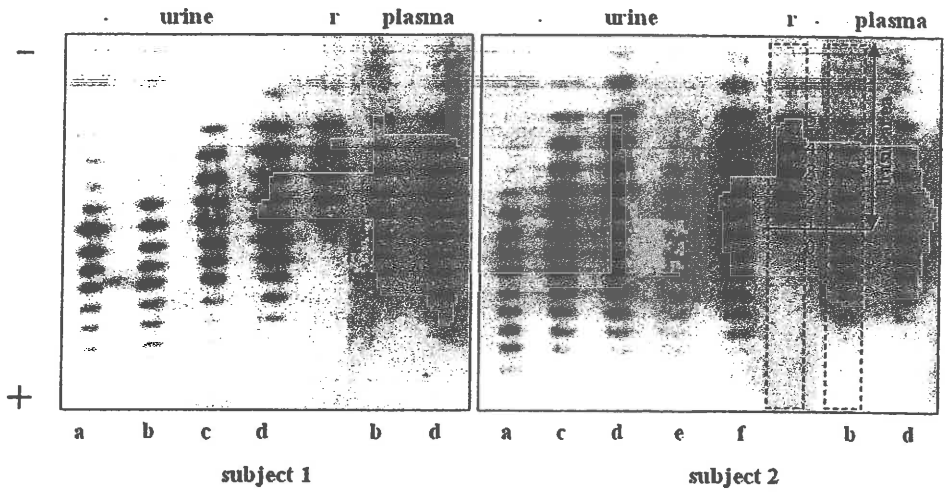

FIGURE 1. IEF patterns of urine and serum EPO from 2 distinct subjects (1 and 2) participating in the protocol: first morning sample (a), immediately before the warm-up training (b), middle (c), and end of exercise (d) and 3 hours after the end of the morning session (e) and end of the afternoon session (f). Lane r corresponds to BRP. Band 1 of BRP is used to define the basic area of the densitometer window (dotted rectangle). Note that b-sample was not collected for subject 2. Note the shift of the bands toward the cathode (top of the figure) induced by exercise. BRP, Biological Reference Preparation; EPO, erythropoietin; IEF, isoelectric focusing.

the sample collected at the end of the exercise is illustrated in Figure 1 for 2 of the 7 subjects tested. Similar results were obtained for all the subjects. Whereas the 2 urine samples collected in the morning before the exercise were very similar (note that no pre-exercise urine was collected for subject 2), a striking shift toward a more basic pI (resulting in more elevated PBI) was observed in the sample collected in the middle of the morning exercise, with no further significant changes at the end of exercise. The observed increase in PBI at the end of exercise compared with the initial values is highly significant ($P < 0.008$, using the Wilcoxon matched-pairs signed-ranks test).

Whenever an additional urine sample was collected 3 hours after the end of the morning session (subject 2 only), a reversal of the pattern to the initial state was clearly initiated but was not completed. A new striking shift toward a more basic pI was observed after the afternoon training session for this particular subject.

In contrast to the urine samples, the corresponding serum samples did not show any shift induced by exercise. The EPO patterns did not undergo any significant change before and after physical exercise (Figure 1).

Before the effort, the isoelectric patterns in all subjects were more acidic for the urine samples than for the serum samples. A significant positive correlation ($r = 0.85$, $P = 0.0158$) was observed between the values of PBI in serum and urine (Figure 2). This correlation, illustrated by the results obtained with the 7 subjects of the experiment, was confirmed by our findings using 47 paired urine and serum samples from other subjects at rest (data not shown).

This correlation remained after exercise ($r = 0.94$, $P = 0.0013$). Moreover, because of the shift toward basic pI, the urine patterns became almost identical to the corresponding serum patterns, as shown by the y intercept at zero point of the regression line in the plot of PBI in serum and urine (Figure 2).

Finally, all generated atypical patterns met at least one of the World Anti-Doping Agency (WADA) positivity criteria. However, none of them fulfilled the 3 criteria mandatory for reporting an adverse analytical finding.

Total Protein, RBP, and EPO Concentrations Prior, During, and After the Effort

A slight increase of EPO concentration was measured in the pre-exercise samples compared with the first morning urine, with no significant changes in total protein and RBP concentrations. A very important increase of the 3 concentrations was observed in the midexercise samples. This increase was even more pronounced in the samples collected at the end of the effort.

Total protein and EPO concentrations increased 8.5- and 10-fold, respectively, compared with the initial values. The

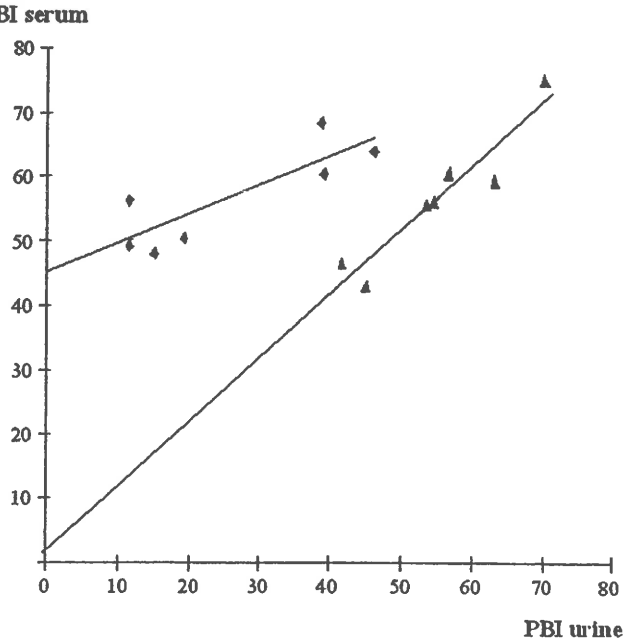

FIGURE 2. Relationship between serum and urine PBI: The regression lines show that PBI is lower in urine before exercise (a-samples) (♦) and becomes equivalent to serum PBI after exercise (d-samples) (▲). PBI, percentage of basic isoforms.

most dramatic rise was observed in the case of RBP, with more than a 300-fold increase at the end of exercise, which incidentally coincided with the maximal shift of the urinary EPO patterns (maximal PBI) (Table 1).

The return to the initial state of the EPO pattern in subject 2, initiated 3 hours after the morning session, was accompanied by a marked decrease in total protein and RBP levels. A second increase in total protein and RBP concentrations was observed again after the afternoon session, which also caused a new shift of the pattern toward a more basic pI. Figure 3 illustrates the similar evolution of the 3 parameters for this particular subject.

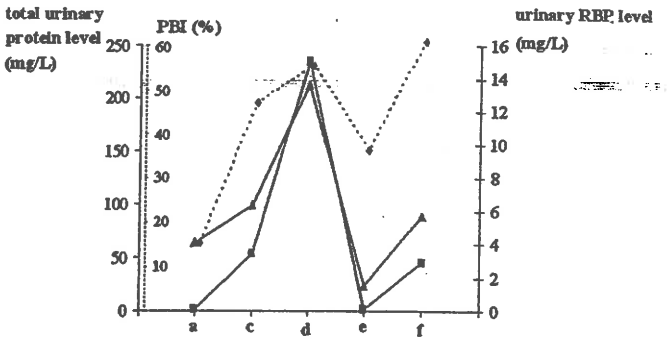

FIGURE 3. Evolution of PBI, urinary total protein, and RBP levels in the case of the athlete who performed 2 training sessions during the day (subject 2). Note that no b-sample was collected. Analogous variations in PBI (♦), urinary RBP (■), and total protein (▲) are observed throughout the entire day. Note the similar evolution of the 3 parameters. PBI, percentage of basic isoforms; RBP, retinol-binding protein.

DISCUSSION

Based on the observations made by Paris and Lausanne anti-doping laboratories, effort EPO patterns are typically observed in urine samples collected after short and intense efforts. Both laboratories have been regularly involved in analyses of samples collected at the completion of stage cycling races. Notably, the occurrence of effort urines increases strikingly after short stages against the clock. Athletics events such as 400 to 3000 m races also seem to frequently generate effort patterns. For example, of the 68 urine samples collected for EPO anti-doping control during the International Association of Athletics Federation indoor world championships 2008 (at the completion of the 400, 800, 1500, and 3000 m races), 55 samples (80%) yielded EPO patterns with PBI above 54% (mean PBI in 38 samples from control subjects at rest: 28.6%; SD, 12.4%), presenting the shift that is characteristic for effort urine. However, it cannot be excluded that other types of effort can also lead to similar shifts. For that reason, the presented explanations should be considered as a hypothesis limited to the range of our observations.

The first goal of this study was to mimic this type of strenuous exercise to produce effort urines under controlled conditions. The protocol we devised was clearly effective in that respect for all the tested subjects who accomplished 3 to 4 series of 30-second effort at their maximal possibilities. In fact, a switch in the EPO patterns was already observable after 2 series. This striking EPO transformation in the urine is clearly related to postexercise proteinuria, as shown by the dramatic increase in urinary protein concentration correlated with increase in PBI. These findings corroborate the fact that postexercise proteinuria is affected by the intensity of exercise rather than its duration and that intermittent effort has a particularly significant impact.[10,11] To our knowledge, the observed quantitative and qualitative variations of urinary EPO have never been described previously. The relation between EPO and total urinary protein is particularly well illustrated in the case of the cyclist who performed 2 training sessions. In this case, EPO concentration and isoelectric patterns evolved in parallel with urinary protein concentration throughout the entire day. The clear return to the basal state of both EPO and protein concentrations initiated 3 hours after the end of the first training session seems to reflect a rapid normalization process that will be investigated in the future.

The important increase in urinary RBP highlights the tubular component of the mixed-type proteinuria associated with strenuous exercise.[12] The concomitant increase in the levels of urinary EPO, which is also a low–molecular weight (30.4 kDa) protein, is probably related to the same mechanism involving both an increase in glomerular permeability and an inhibition of proximal tubular reabsorption. The striking transformation of its isoelectric pattern induced by exercise is, however, more surprising. Clearly, as shown by the stable isoelectric patterns, exercise does not modify the isoforms of serum EPO. Based on isoelectric patterns obtained in earlier work[8] and the present study, physiological urinary and serum EPOs seem to differ at rest, the former being systematically more acidic than the latter. Strenuous exercise obliterates this difference, yielding urinary patterns that are very similar to serum patterns. This finding may be explained by the fact that under resting conditions, most of postglomerular filtered EPOs are absorbed by proximal tubule cells. Indeed, the recognition and the binding of low–molecular weight proteins by the brush border membranes of tubule cells have been shown to be strongly influenced by the electrical charge of proteins.[13] Other

TABLE 1. Relative Increases of PBI, RBP Levels, Urinary Total Protein, and EPO Levels in Urine: First Morning Urine Sample (a), Immediately Before the Warm-up Session (b), Middle (c), and End of the Exercise (d)

	b/a	c/a	d/a	
μ	0.90	2.04	2.31	PBI
SD	0.11	0.74	0.93	
μ	1.54	143.17	332.64	RBP
SD	0.39	113.28	311.24	
μ	2.34	6.06	8.50	Total protein
SD	1.25	3.39	3.94	
μ	2.09	5.19	10.02	EPO
SD	0.67	5.49	3.99	

For each subject, relative increases are calculated as the ratio of the observed values at different times to the corresponding basal values in (a).

EPO, erythropoietin; PBI, percentage of basic isoforms; RBP, retinol-binding protein; μ, mean ratios.

experiments using different modified albumin molecules have shown that the binding to brush border membranes increases with the pI.[14] Thus, it is conceivable that the most basic isoforms of serum EPO, after filtration by the glomerulus, are preferentially removed from the urine by tubular cells, thus yielding a more acidic isoelectric pattern in the collected sample. If so, and if the tubular reabsorption process is saturated, such as after a strenuous exercise, the basic isoforms cannot be removed anymore and the serum EPO isoelectric pattern remains unchanged in the urine.

Whatever the reason of the basic shift observed in EPO isoelectric patterns is, this phenomenon has to be taken into account when interpreting the result of an anti-doping control. To prevent any misinterpretation that could lead to a false-positive result, the current positivity criteria for urinary rhEPO, as referenced in the last version of the WADA technical document (TD2007EPO),[7] take into account the existence of effort urines and are therefore very conservative. Hence, the fact that all atypical patterns met at least one of them constitutes a complete justification of the use of these criteria. The drawback in such a careful approach is that low doses of recombinant EPO in urine cannot be reported, yielding false-negative results.[15] It should be possible to use criteria that are more flexible, with reliable reporting of lower doses of recombinant EPO, in cases where the opportunity of a shift due to physical exercise can be categorically ruled out. Finally, our results indicate that it might be possible to use the urinary RBP level as a marker to identify urine samples in which the positivity criteria can be relaxed. This RBP dosage could be possibly combined with complementary methods, such as a sodium dodecyl sulfate polyacrylamide gel electrophoresis test. Indeed, this last recently seemed to be beneficial and reliable in relation to active and effort urines.[16,17] Further investigations are, however, necessary to test this possibility.

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