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A New Principle Suggested for Detection of Darbepoetin-α (NESP) Doping

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ABSTRACT

Doping with darbepoetin-α, also termed novel erythropoiesis stimulating protein (NESP), a hypersialylated, very effective analogue of erythropoietin, is a serious threat in sport. We report here on a new principle for the detection of darbepoetin-α in serum based upon increase in immunoactivity after desialylation with neuraminidase. The method is evaluated on sera from patients taken 2–14 days after last injection of darbepoetin-α.

Thirty-two venous blood samples and 3 capillary samples taken from finger tips were obtained from 13 patients with end stage renal disease treated with intravenous or subcutaneous injections of Aranesp, 0.45 to 2.60 $\mu g*kg^{-1}$. Blood samples from 37 individuals with endogenous erythropoietin were used as controls. The sera were diluted 1:2 with acetate buffer pH 5.6 with or without neuraminidase and incubated at 37°C for 1 or 24 h before immunoassay. The erythropoietin immunoactivity in serum volumes of 12.5–50 µL was measured with ELISA-kits from R&D Systems Inc and medac GmbH.

The relative increase in immunoactivity after desialylation was in all cases higher for the darbepoetin-α samples than for any of the control samples assayed in parallel, varying incubation time with the enzyme, serum volumes and batches of both ELISA-kits. The mean relative increase in immunoactivity of endogenous erythropoietin after neuraminidase was 42 % with the medac-kit and 117 % with R&D-kit while the corresponding figures for darbepoetin-α were 282 % and 231 % with 1 h and 299 % and 256 % with 24 h enzyme incubation, respectively. Endogenous and recombinant human erythropoietin showed similar relative increase after desialylation.

The method to detect darbepoetin-α in serum is simple to perform, robust, sensitive and requires a small amount of blood. The drug was detected in all patient sera taken 2–14 days after last injection. We suggest that the method should be evaluated for the detection of darbepoetin-α doping in sport.

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INTRODUCTION

Darbepoetin-α, also termed novel erythropoiesis stimulating protein (NESP), is an analogue of recombinant human erythropoietin (rHuEPO). The polypeptide backbone differs at five amino acid positions and the molecule has five N-linked carbohydrate chains, compared to three on rHuEPO (1). The two extra carbohydrate chains with terminal sialic acid groups prolong its serum half-life which increases its biological activity (2). Darbepoetin-α is effective in treatment of anaemia in patients with chronic kidney disease and when administered once weekly or once every other week the effect is similar to rHuEPO given three times a week (3). The greater biological activity and lower dosing frequency compared with rHuEPO makes darbepoetin-α very attractive for clinical use but, unfortunately, also attractive for doping in endurance sports. In fact, soon after darbepoetin-α was approved for medical use, the abuse of the drug was disclosed at the 2002 Winter Olympic Games in Salt Lake City. Cross-country ski team members from Russia and Spain were disqualified and stripped of their gold medals.

Already 15 years ago, when rHuEPO became available, it was obvious that it represented a major threat to fair competition in endurance sports. EPO was classified as a doping substance by the International Ski Federation in 1988 and by the International Olympic Committee in 1990; although at that time no method existed to detect it in body fluids. The same year we reported that rHuEPO was less negatively charged than endogenous EPO in serum and that the two compounds could be separated by electrophoresis (4). Based upon these observations we described in 1995 a method for detection of rHuEPO in serum and urine (5). In 2000, Lasne and de Ceaurriz (6) described an isoelectric focusing (IEF) method combined with immunoblotting to observe the difference in charge and trace rHuEPO in urine. Neither of these methods for the detection of rHuEPO is suitable for screening in large series. They are time consuming, expensive, labour intensive, require well trained technicians and can only be performed in specialized laboratories.

There is hitherto only one report on the detection of darbepoetin-α in body fluids. Catlin et al. demonstrated that they could detect darbepoetin-α in a urine sample from a cancer patient one week after administration of a single dose (0.675 $\mu g \times kg^{-1}$) of Aranesp® (Amgen Inc.) (7). They concentrated the urine 1000 times and used the IEF method with double-blotting as described by Lasne (8) to detect darbepoetin-α which was more negatively charged than endogenous EPO.

We here report a new principle for detection of darbepoetin-α based upon the change of immunoactivity after treatment with neuraminidase. The enzyme removes the sialic acid residues and after this treatment the immunoactivity of darbepoetin-α, with its higher content of sialic acid, increases considerably more than that of endogenous EPO or rHuEPO. This observation forms the basis for a simple assay method using serum and is suggested suitable for use for detection of doping with darbepoetin-α. The serum sample is incubated at 37˚C with or without the enzyme and then analyzed for EPO immunoactivity with a commercially available test-kit. The analyses require small amounts of blood (50–100 μL serum for a complete test)

Table 1. Data on patients with end stage renal disease treated with intravenous (iv) or subcutaneous (sc) injections of darbepoetin-α (Aranesp®) and day of sample collection.

		Administration of Aranesp®				
Patient Sex	Age: years	Treatment months	Frequency interval, days	Injection	Dose $\mu g \times kg^{-1}$	Sample collection Day after last injection
Female	73	3	7	iv	0.59	5, 7
Male	40	3	7	iv	0.60	7, 7, 7, 7
Male	29	3	7	iv	0.68	7, 7
Female*	30	0	7	sc	0.45	7
Female*	67	0	7	sc	0.46	7
Female	77	6	7	sc	0.51	2, 7
Male	84	38	7	sc	0.76	2, 7, 7, 7
Female	21	10	7	iv	1.32	7, 7, 7
Male	88	4	7	iv	1.44	4, 7,7, 7
Male	79	4	14	iv	1.49	7, 12, 14, 14
Female	90	15	14	iv	1.57	5, 7
Male	20	10	14	iv	1.68	2, 9
Female	35	3	14	sc	2.60	14

*) not yet started on haemodialysis therapy

and it is possible to obtain the results within a few hours. The method is easy to perform and suitable for screening of large series of serum samples. The method was evaluated on analyses of blood samples from patients with chronic renal disease treated with darbepoetin-α (Aranesp®, Amgen).

SUBJECTS AND METHODS

Subjects

Thirty-two venous blood samples were obtained from 13 patients with end stage renal disease treated with darbepoetin-α (Aranesp®, Amgen). Eleven of the patients were on haemodialysis therapy. Sex, age, administration of Aranesp®, and day of sampling after last injection, are given in Table 1. Capillary blood samples were also taken from the finger tip of three patients 7 days after an intravenous injection of Aranesp. The participants got written information and gave their consent. Sera with endogenous EPO levels comparable with those of the darbepoetin-α group were used as controls. Thirty of these were from patients with various diseases but not treated with darbepoetin-α and seven were from healthy volunteers working in the laboratory. The control group consisted of 20 men, 22–77 years and 17 women, 18–73 years.

Methods

The EPO immunoactivity was measured with two commercial test kits for serum EPO, the "medac-kit": EPO-ELISA medac; medac GmbH, Hamburg, Germany and

the "R&D-kit": Quantikine IVD Erythropoietin ELISA; R&D Systems Inc, Minneapolis, USA. The assays were performed according to the protocols of the manufactures, with the exceptions that the serum volumes were smaller and that before assay the sera were diluted 1:2 in 0.2 M acetate buffer pH 5.6 and incubated for 1 or 24 h at 37°C with or without neuraminidase (from Arthrobacter ureafaciens, Sigma-Aldrich Chemie or ICN Biomedicals) 140 $mU \times mL^{-1}$. The preincubations were made in test tubes from which two aliquots were taken for the EPO assay. The results were expressed in $IU \times L^{-1}$ using the standards supplied with the kits. The estimated serum concentrations of EPO and darbepoetin-α after dilution 1:2 with the acetate buffer, without the enzyme, and incubated for various times at 37°C, were close to those obtained with the protocols of the kit manufactures.

An rHuEPO preparation (the Int Stand for EPO, Recombinant DNA-Derived; WHO 87/684)[9] was added to sera with low endogenous EPO concentration and the relative increase of immunoactivity after neuraminidase treatment was measured with both EPO-kits.

RESULTS

A total of 151 determinations of increase of immunoactivity after desialylation were made during a 13 months period on the 35 samples from the darbepoetin-α treated

Table 2. Relative increase in immunoactivity of darbepoetin-α and endogenous erythropoietin (EPO) after neuraminidase treatment for 1 or 24 h measured with two different serum EPO ELISA-kits. The sera with darbepoetin-α were taken 2–14 days after injection of Aranesp®.

EPO-kit	Enzyme treatment h	Serum samples with	Number of samples	Serum volume µL	Immunoactivity $IU*L^{-1}$ mean	Immunoactivity $IU*L^{-1}$ range	Per cent increase in activity after enzyme treatment mean	Per cent increase in activity after enzyme treatment range
medac GmbH	1	EPO	30	12.5	22	10–80	42	5–77
medac GmbH	1	darbepoetin-α	30	12.5	28	5–78	282	157–454
medac GmbH	24	EPO	32	12.5	18	8–50	43	0–79
medac GmbH	24	darbepoetin-α	32	12.5	24	6–118	299	169–465
R&D Systems Inc	1	EPO	29	25	26	11–68	118	81–135
R&D Systems Inc	1	darbepoetin-α	29	25	40	8–174	231	150–315
R&D Systems Inc	24	EPO	30	50	25	5–54	116	87–149
R&D Systems Inc	24	darbepoetin-α	30	50	40	10–155	256	159–373

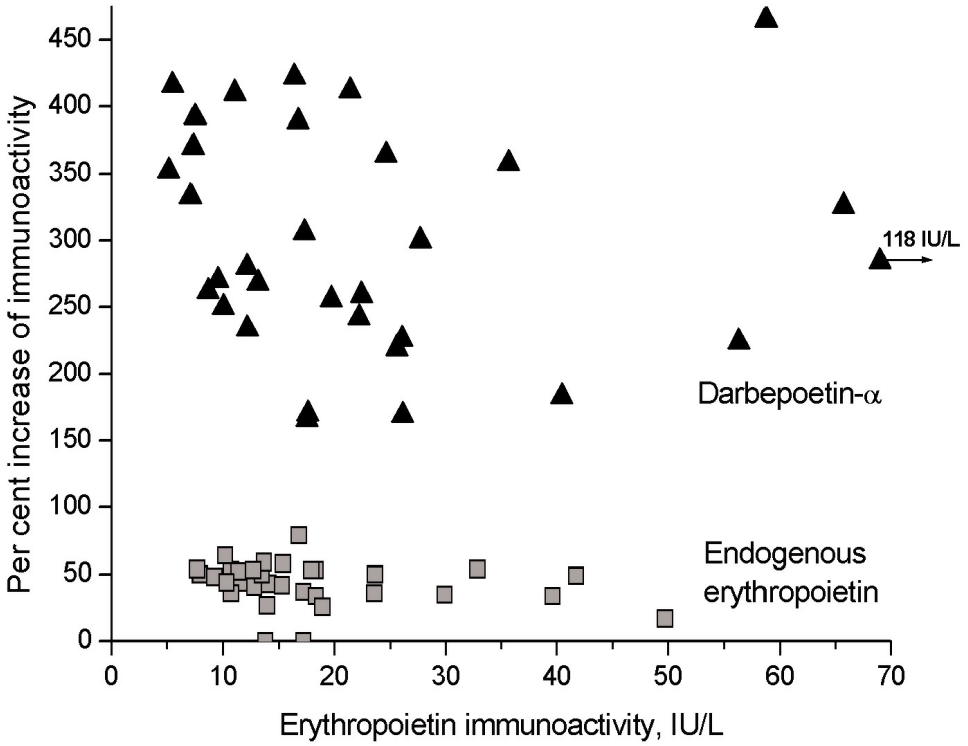

Fig. 1. Relative increase in immunoactivity after 24 h neuraminidase treatment of 32 sera with darbepoetin-α (▲) and 32 with endogenous erythropoietin (■) in relation to the serum EPO concentration analysed in 12.5 µL serum with an EPO-ELISA-kit (medac). The sera with darbepoetin-α were taken 2–14 days after injection of Aranesp,.

patients. These studies included changes in variables such as the amount of serum assayed from 12.5 to 50 µL, the enzyme incubation time from 1 to 24h and the use of 6 batches of the R&D-kit and 3 of the medac-kit. The relative increase in immunoactivity after desialylation was in all cases higher for the darbepoetin-α samples than for any of the 37 control samples assayed a total of 150 times in parallel. A comparison of the results from preincubation with the enzyme for 1 h or 24 h using the two different test-kits is shown in Table 2. The mean relative increase in immunoactivity of endogenous EPO after neuraminidase was about 42 % with the medac-kit and 117 % with R&D-kit while the corresponding figures for darbepoetin-α were 282 % and 231 % with 1 h enzyme incubation and 299 % and 256 % with 24 h enzyme incubation, respectively.

The relationship between the serum EPO concentration measured with the medac-kit and the relative increase in immunoactivity after enzyme treatment of 32 sera with darbepoetin-α and 32 with endogenous EPO is shown in Figure 1. The preincubation time with neuraminidase was 24 h and the amount of serum assayed 12.5 µL. There was no significant correlation (P>0.05) between the serum concentration and the relative increase for any of the compounds.

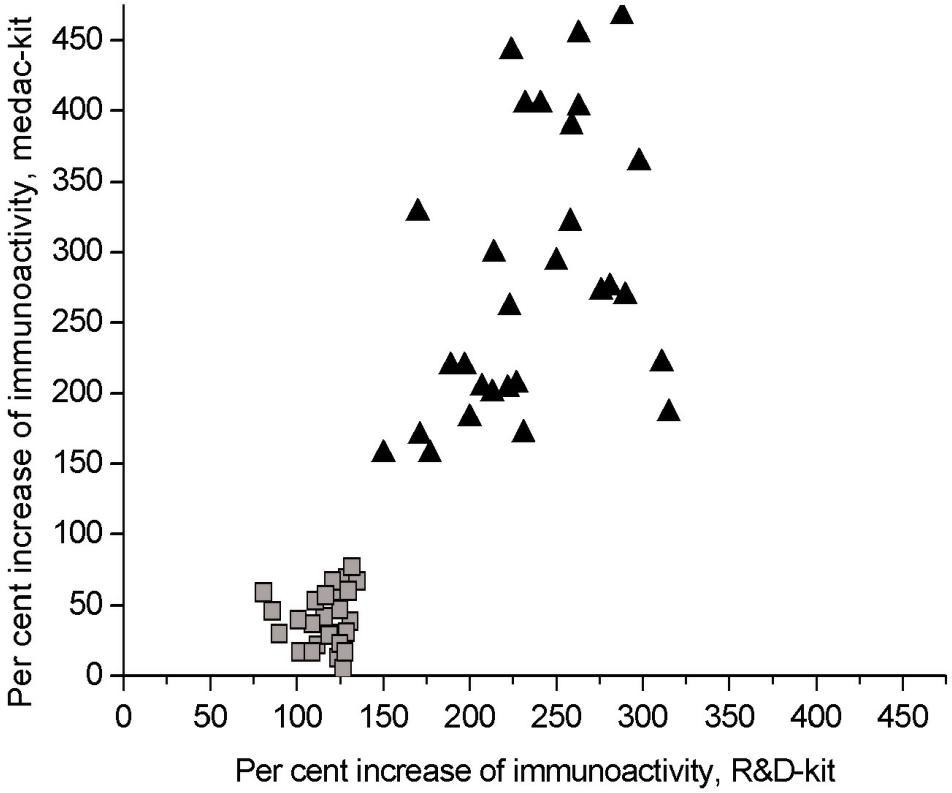

Fig. 2. Relative increase in immunoactivity after 1 h neuraminidase treatment of 29 sera with darbepoetin-α (▲) and 26 with endogenous erythropoietin (■). Comparison of results obtained with EPO ELISA-kits from R&D Systems Inc and medac GmbH, analysed in aliquots of 25 and 12.5 µL of serum, respectively. The sera with darbepoetin-α were taken 2–14 days after injection of Aranesp,.

The power to discriminate between the darbepoetin-α and the EPO groups by the responses to neuraminidase treatment was greater with the medac-kit than with the R&D-kit. This is illustrated in Figure 2 using 1 h enzyme incubation and in Figure 3 using 24 h enzyme incubation. The results with R&D-kit were on the other hand more reproducible with hardly any outliers. The within-assay coefficient of variation, estimated from assays in duplicate, was 3.04 % (n=136) with the R&D-kit and 6.19 % (n=188) with the medac kit.

Most of the sera were re-assayed 4 to 8 times over periods of several months. The sera were stored at –20˚C between assays, without any detectable change in immunoactivity, with or without neuraminidase treatment. Also the activities in sera diluted with acetate buffer and incubated with or without the enzyme were stable when stored at –20˚C.

The test requires very small amounts of serum (60 µL for 4 × 12.5 µL assays) and therefore the use of capillary blood samples taken from the finger tip was also investigated. The results were similar to those obtained with venous blood samples

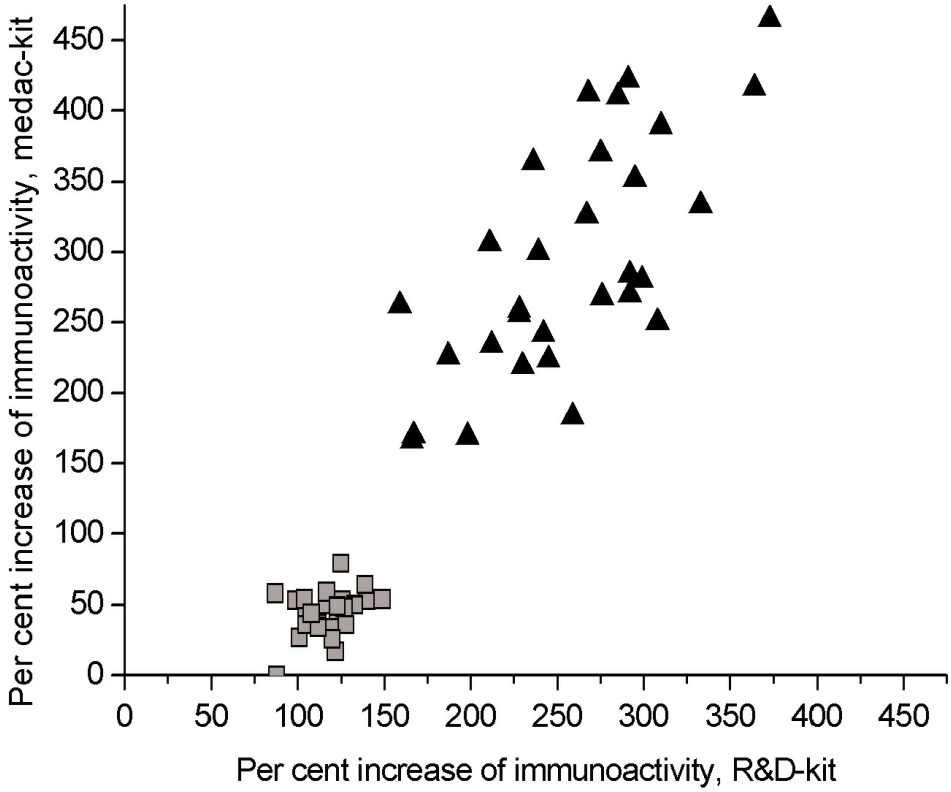

Fig. 3. Relative increase in immunoactivity after 24 h neuraminidase treatment of 30 sera with darbepoetin-α (▲) and 28 with endogenous erythropoietin (■). Comparison of results obtained with EPO ELISA-kits from R&D Systems Inc and medac GmbH, analysed in aliquots of 50 and 12.5 µL of serum, respectively. The sera with darbepoetin-α were taken 2–14 days after injection of Aranesp,.

and the percentage increases in immunoactivity after enzyme treatment using the medac-kit were: 29, 48 and 52 for 3 control individuals and 265, 297 and 388 for 3 patients treated with Aranesp®.

The relative increase of immunoactivity after desialylation of rHuEPO added to serum was similar with that of endogenous EPO. The percentage increases of immunoactivity for rHuEPO added to two sera were 39 and 68 using the medac-kit and to three sera using the R&D-kit 107, 116 and 120, respectively.

DISCUSSION

Darbepoetin-α was detected in all blood samples taken 2–14 days after intravenous or subcutaneous injections by the higher increase in immunoactivity after neuraminidase treatment. In the present study the blood samples with darbepoetin-α were from patients with kidney disease and not from athletes which would be the

case if the method is used for doping control. If the kidneys play an important role for the degradation of darbepoetin-α, it could be expected that in these patients with kidney disease, an accumulation of the drug in serum may occur after long treatment. However, pharmacokinetic studies have not given any evidence for such an accumulation in patients with renal failure given darbepoetin-α intravenously or subcutaneously once weekly (10). The metabolic fate of circulating native EPO, rHuEPO and darbepoetin-α is still an enigma. Jelkman (11) concluded in a review that the liver and the kidneys do not seem to play a major role in the degradation and that the majority of EPO and its analogues most likely are degraded following EPO-receptor-mediated uptake in the bone marrow. The pharmacokinetic profile of darbepoetin-α in patients with cancer was reported to be similar to that in patients with chronic kidney disease (12).

The differences observed in measured activity between the two EPO-kits are most likely due to use of different antibodies directed against different epitopes on the molecules. Both EPO-kits used in this study are sandwich ELISA techniques using wells pre-coated with murine monoclonal antibodies against human EPO. The enzyme labelled antibodies are murine monoclonal in the medac-kit and rabbit polyclonal in the R&D-kit. The increased immunoactivity after neuraminidase treatment is most likely an effect of changes in the tertiary structure of the molecules facilitating a better antibody-epitope binding.

The power to discriminate between the darbepoetin-α group and the control group by the increase of immunoactivity after neuraminidase treatment was similar after 1 and 24 h incubation. The activity of desialylated darbepoetin-α was slightly higher after 24 h compared with 1 h incubation. With electrophoretic analyses of darbepoetin-α in serum we found that, with the neuraminidase concentration used in the present study, the desialylation was incomplete after 1 h compared with 24 h (unpublished observation). From a practical point of view, we suggest that use of overnight incubation (about 16 h at 37°C) with neuraminidase and then start the EPO-assays in the morning may be a suitable routine for many laboratories.

The blood tests to detect EPO doping in use at present are presumption tests based upon measurements of four parameters: haemoglobin and percent reticulocytes in the blood and total (endogenous and exogenous) serum EPO and serum soluble transferrin receptor (13). The serum EPO tests are based on ELISA formats, like in the present study, and we suggest an extra analysis of the serum after neuraminidase treatment to reveal darbepoetin-α doping. It may sometimes be preferable to use capillary blood samples taken from the finger tip. We have found that volumes of 500–600 μL blood are enough for repeated analyses for detection of darbepoetin-α in serum. This may be useful in out-of-competition doping controls.

The main clinical advantage of darbepoetin-α compared with rHuEPO is the lower dosing frequency, once a week or once every other week. The adverse effects of darbepoetin-α are similar to those of rHuEPO (3), but hitherto, the serious compli-

cation with pure red cell aplasia associated with antibodies during treatment with rHuEPO (14), has not been reported for darbepoetin-α. If the clinical success of the drug in patients with anaemia due to kidney disease or cancer continues and the number of clinical applications increases, as in the case of rHuEPO (15), darbepoetin-α becomes more generally available with increased risk of abuse in sport. The method described above for the detection of darbepoetin-α is simple to perform at any clinical chemical or doping laboratory. In this study the drug was detected in serum 2–14 days after injection. We suggest that the method should be evaluated for detection of darbepoetin-α doping in sport.

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