



ELSEVIER

Journal of Immunological Methods 294 (2004) 101–110

JIM
Journal of
Immunological Methods

www.elsevier.com/locate/jim

Research paper

Development and evaluation of a new ELISA for the detection and quantification of antierythropoietin antibodies in human sera

W. Hoesel^{a,*}, J. Gross^b, R. Moller^b, B. Kanne^a, A. Wessner^a, G. Müller^f, A. Müller^f,
E. Gromnica-Ihle^c, M. Fromme^d, S. Bischoff^e, A. Haselbeck^a

^aRoche Diagnostics GmbH, Nonnenwaldstr. 2, 82372 Penzberg, Germany

^bDepartment of Otorhinolaryngology, Molecular Biological Research Laboratory, Humboldt University,
Charité Hospital, Spandauer Damm 130, 14050 Berlin, Germany

^cBerlin-Buch Rheumatology Clinic, Karower Str. 11, 13125 Berlin, Germany

^dWolframstr. 60-62, 70191 Stuttgart, Germany

^eCaspar-David-Friedrich Str. 10a, 01217 Dresden, Germany

^fMicrocoat Biochemische Produkte GmbH, Am Neuland 3, 82348 Bernried, Germany

Received 8 June 2004; received in revised form 3 August 2004; accepted 31 August 2004

Available online 4 October 2004

Abstract

Assays for the analysis of antierythropoietin antibodies (anti-EPO Abs) currently suffer from a high degree of nonspecificity or are cumbersome and time consuming to perform. They are therefore not well suited for the analysis of large numbers of human sera samples, a task that has become increasingly important due to an increase in the number of patients developing anti-EPO Abs. The objective of this study was to develop and validate a sensitive and specific ELISA for the determination of anti-EPO Abs that would suit these purposes.

In this new double antigen bridging ELISA, anti-EPO Abs bind via one site to recombinant human erythropoietin (rhEPO)-biotin immobilized to streptavidin-coated microtiter plates (MTPs) and by a second site to rhEPO labelled with digoxigenin (DIG). The amount of bound antibody is determined using an anti-DIG antibody coupled to peroxidase. A rabbit polyclonal anti-EPO Ab purified by immunoabsorption is used as reference antibody preparation.

The dynamic range of this ELISA was 1–75 ng/ml per assay calibrated with the reference antibody preparation. The assay was specific for anti-EPO Abs and did not react with other immunoglobulins (Ig) present in human serum. The lower limit of detection (LLD) of the assay was 0.5 ng/ml, and the lower limit of quantitation (LLQ) was 1.0 ng/ml. Anti-EPO Abs could be detected in the sera of pure red cell aplasia (PRCA) patients. In contrast to previous reports, no anti-EPO Abs could be

* Corresponding author. Tel.: +49 8856 603274; fax: +49 8856 603341.

E-mail address: wolfgang.hoesel@roche.com (W. Hoesel).

detected in the sera of patients with systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), Sjögren's syndrome (SS), or in the sera of dialysis patients.

© 2004 Elsevier B.V. All rights reserved.

Keywords: Antierythropoietin antibodies (anti-EPO Abs); ELISA; PRCA; Autoimmune diseases

1. Introduction

Recombinant human erythropoietin (rhEPO) is a sialoglycoprotein hormone with an action that is the biological equivalent of endogenous erythropoietin (EPO). It is used in the clinic to stimulate red blood cell development in patients with anemia due to kidney failure or during treatment for cancer (Dunn, 1996; Besarab and Erslev, 1997). In the last few years, there has been an increase in the number of patients developing anti-EPO antibodies (Abs) during the course of therapy with rhEPO, and most of these individuals developed pure red cell aplasia (PRCA; Casadevall et al., 1996, 2002; Bunn, 2002; Casadevall, 2002; Gershon et al., 2002; Weber et al., 2002). The increase in anti-EPO Ab-positive patients developing PRCA has been mainly associated with the use of one rhEPO brand, namely, EPREX[®], after its formulation was changed in 1998 (Casadevall, 2003; Hermeling et al., 2003). These incidences of PRCA have triggered a large increase in the interest in, and an urgent need for, the detection and measurement of antierythropoietin antibodies (anti-EPO Abs). Four types of assays have currently been described and used for the analysis of anti-EPO Abs: (A) a radio-immunoprecipitation assay (RIPA) using I¹²⁵ labelled EPO and immobilized protein G from *Staphylococcus aureus* (Casadevall et al., 2002; Tacey et al., 2003); (B) ELISA with a displacement step (Kientsch-Engel et al., 1989; Urra et al., 1997); (C) ELISA without a displacement step (Tzioufas et al., 1997; Sipsas et al., 1999; Castelli et al., 2000; Voulgarelis et al., 2000; Schett et al., 2001); and (D) BIAcore analysis (Swanson, 2003).

A specific and sensitive RIPA has recently been described by Tacey et al. (2003) in some detail. This assay has been used in the seminal investigations of Professor Casadevall's laboratory in which anti-EPO Abs in the sera of PRCA patients were detected (Casadevall et al., 1996, 2002; Casadevall, 2002).

There have been several modifications of the ELISA with displacement reactions. An ELISA described by Kientsch-Engel et al. (1989) uses biotin-labelled rhEPO (rhEPO-Bi) immobilized to streptavidin-coated microtiter plates (MTPs). Anti-EPO Abs bound to rhEPO-Bi are detected by sheep antihuman immunoglobulin (Ig) Abs coupled to peroxidase. This method includes control material to assess the function of the test and uses, as a second step, a competitive displacement reaction where rhEPO is added to the assay in order to distinguish between the detection of specific and nonspecific antibodies. Another ELISA described by Urra et al. (1997) uses MTPs coated with a high concentration of rhEPO (10 mg/l), and a peroxidase goat IgG conjugate specific for the detection of human Ig is used to measure the binding of anti-EPO Abs. To evaluate the specificity of the ELISA, patient serum samples are preincubated with 1.5 mg/l of rhEPO for competitive displacement in a second assay.

The ELISA procedure without displacement basically consists of the first step of an ELISA assay with displacement. Several modifications of this assay have been performed and published. Sipsas et al. (1999) used polystyrene plates coated with rhEPO, and binding of IgG antibodies was indicated with goat antihuman IgG conjugated with alkaline phosphatase. Castelli et al. (2000) also used rhEPO adsorbed to a microtiter plate, but rabbit antihuman Ig labelled with horseradish peroxidase was used to detect anti-EPO Abs.

BIAcore analysis for the detection of anti-EPO antibodies in human serum has been used and described by Swanson (Mason et al., 2003; Swanson, 2003). This method detects the real-time binding of antibodies as an increase in mass accumulating on the rhEPO protein that is immobilized to a sensor chip.

The four test methods described above all suffer from one or more disadvantages. The RIPA and ELISA with displacement methods are cumbersome

and/or time consuming. For example, the use of radioactivity and two days of analysis time are required for RIPA (Tacey et al., 2003), while two assays have to be performed for the ELISA procedure with displacement. Although the displacement ELISA and RIPA yield very specific and sensitive results, this is not true for ELISAs without displacement, especially with regard to specificity. Inasmuch as this assay is based upon the detection of bound Ig and lacks a specific displacement step, it is prone to false positive results. Finally, BIAcore analysis needs specialized equipment that has to be run by experienced people. Its advantage that samples can be analyzed in real time is offset by a certain lack of sensitivity and specificity when analyzing low analyte concentrations in sera, and nonspecific binding of substances to the BIAcore chip surface can cause interference.

Here, we describe the development of a sensitive and specific test that is able to quantify anti-EPO Abs, at least in a relative manner, and that can be used to analyze large numbers of samples in a reasonable time. It was used to analyze anti-EPO Ab-positive samples that had previously been identified by either RIPA (Casadevall et al., 2002; Tacey et al., 2003) or ELISA with displacement (Kientsch-Engel et al., 1989), or had originated from screening programs of anemia patients who were suspected of having anti-EPO Abs. Inasmuch as a high incidence of anti-EPO Ab-positive samples have been reported for patients with systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), Sjögren's syndrome (SS), and those undergoing dialysis (Tzioufas et al., 1997; Sipsas et al., 1999; Castelli et al., 2000; Voulgarelis et al., 2000; Schett et al., 2001), sera from these individuals were used to assess the application of this test.

2. Materials and Methods

2.1. Materials

Microtiter plates precoated with streptavidin were obtained from Microcoat, Bernried, Germany. Anti-EPO polyclonal antibody (PAb; immunosorbed) from rabbit, rhEPO, antidigoxigenin–horseradish peroxi-

dase (anti-DIG–HRP) conjugate, ABTS (2,2'-Azinodi-[3-ethylbenzthiazoline sulfonate(6)] diammonium salt), and all reagents for the incubation buffers of the ELISAs were obtained from Roche Diagnostics, Penzberg, Germany. All other reagents were from Merck, Germany, if not indicated otherwise.

Phosphate buffer (PB; pH 7.2) contained 40 mM potassium phosphate and 0.1% Tween 20. HBe buffer was used as the incubation buffer of the HBe ENZYMUN[®] test (Roche Diagnostics).

Twenty sera from rhEPO-treated patients who were suspected of having anti-EPO Abs were obtained from nephrologists and several dialysis centers from Germany and abroad. Control (anti-EPO Ab-negative) serum samples for the cut-off determination were collected from blood banks, dialysis centers, and hospitals by Roche Diagnostics. Sera from patients with SLE ($n=51$; 18–69 years), RA ($n=94$; 26–80 years) and SS ($n=12$; 32–72 years) were collected from the Berlin–Buch Rheumatology Clinic and stored at -80°C until analysis.

2.2. Methods

2.2.1. Preparation of biotinylated rhEPO

Biotin was linked to rhEPO in two different ways. One derivative was prepared by coupling biotin to amino groups of rhEPO, using biotin- ϵ -aminocaproic acid-*N*-hydroxy-succinimide ester (rhEPO-Bi-X-NHS). The other derivative was prepared using biotin- ϵ -aminocaproic acid-hydrazine to link the biotin to carbohydrate groups of rhEPO (rhEPO-Bi-X-H). Both reagents were prepared according to standard protocols (Haselbeck and Hoesel, 1999; Peter et al., 1999), and a mixture in the ratio 2:3 was used for coating the streptavidin MTPs.

2.2.2. Preparation of rhEPO-DIG

Digoxigenylation of rhEPO was performed using digoxigenin- ϵ -aminocaproic acid-*N*-hydroxy-succinimide ester (DIG-X-NHS), according to a standard protocol (Peter et al., 1999).

2.2.3. Preparation of PAb anti-EPO-(R)-IgG-(IS)

The polyclonal anti-EPO IgG fraction from rabbits was immunosorbed on an EPO-Sepharose column and eluted by 1 M propionic acid according to standard procedures.

2.2.4. Anti-EPO double antigen sandwich assay

A mixture of rhEPO-Bi-X-H (0.75 $\mu\text{g/ml}$) and rhEPO-Bi-X-NHS (0.5 $\mu\text{g/ml}$) in HBe buffer was incubated in a streptavidin-coated MTP at a final volume of 125 $\mu\text{l/well}$ for 30 min. The wells were then washed three times with PB. Human control serum diluted 1:5 with HBe buffer or reference antibody preparation (rabbit polyclonal anti-EPO Ab) diluted in human control serum/HBe buffer (1:5; v/v) were mixed with an equal volume of DIG-labelled rhEPO (dissolved at 40 ng/ml in HBe buffer). This solution (100 μl) was added to the wells without delay and then incubated for 2 h. After washing three times with PB, 100 μl of anti-DIG Fab fragments–HRP conjugate (75 mU/ml) freshly prepared in HBe buffer was added to each well and then incubated for 1 h. After washing (three times with PB), 100 μl ABTS solution (1 mg/ml in ABTS buffer) was added to each well and incubated for 30–60 min. Absorbance values were measured at 405 and 492 nm for reference. The calibration curves were calculated using the 4-parameter regression curve. All incubations of the MTPs were performed at room temperature while shaking at 300 rpm and with the MTP sealed by a plastic cover. The overall turnaround time for this ELISA was in the order of 4 h.

The anti-EPO two-step ELISA with displacement was used in the present study to provide a reference. The methods of this ELISA have been described in detail elsewhere (Kientsch-Engel et al., 1989). Using this assay, the sera of 20 PRCA patients from different clinics had been unequivocally identified to contain anti-EPO Abs. The presence of anti-EPO Abs in 19 of these samples was further confirmed in the lab of Casadevall et al., using the RIPA assay (Tacey et al., 2003). The other patient has been described in detail previously (Weber et al., 2002).

3. Results

3.1. Assay development

The principle of this newly developed ELISA is that anti-EPO Abs bind with one site to biotinylated rhEPO immobilized to streptavidin-coated MTPs and with a second binding site to digoxigenin-labelled rhEPO. The amount of Ab bound is determined colorimetrically using an anti-DIG Ab coupled to horseradish peroxidase and ABTS as a substrate (as shown in Fig. 1), and a rabbit polyclonal anti-EPO Ab is used as a calibrant.

For the most critical reagents in the assay, concentrations of 1.25 $\mu\text{g/ml}$ for rhEPO-Bi and 0.02 $\mu\text{g/ml}$ for rhEPO-DIG were determined as optimal. In the case of the rhEPO-Bi, a mixture of biotinylated EPO linked via amino and carbohydrate groups was used for the coating in order to provide as much accessibility of the EPO at the wall surface as possible. A typical standard curve was obtained for the ELISA using different concentrations of the reference Ab preparation and is presented in Fig. 2. The standard assay detected a range of concentrations of this material from 1 to 75 ng/ml (final concentrations in the well). Further characteristics of the assay were determined as follows. The lower limit of detection (LLD) was estimated at 0.5 ng/ml (3 S.D.; $n=8$) and the lower limit of quantitation (LLQ) at 1.0 ng/ml (6 S.D.; $n=8$). The intra-assay variability (CV) was below 6% at all concentrations used ($n=8$). Day-to-day variation (interassay imprecision; CV) was assessed by two individuals analyzing the standards and controls on four different days, with the reagents prepared freshly each day. Interassay variability for all standard concentrations was below 6%. The CV of a human serum sample containing a low concentration

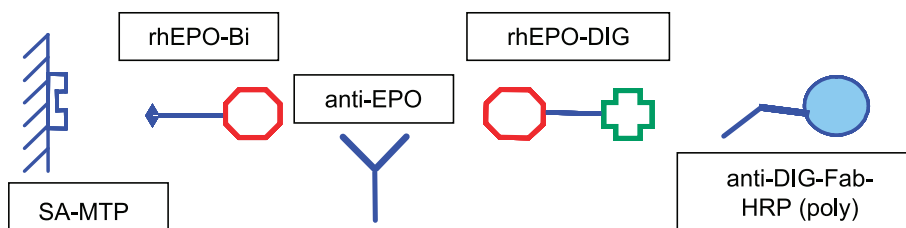


Fig. 1. Principle of the anti-EPO double antigen bridging assay for the analysis of human sera. rhEPO-Bi—biotin-labelled recombinant human erythropoietin; rhEPO-DIG—digoxigenin-labelled recombinant human erythropoietin; SA-MTP—streptavidin-coated microtiter plate; anti-DIG-Fab–HRP(poly)—anti-digoxigenin Ab (Fab fragments) conjugated with polymerized horseradish peroxidase.

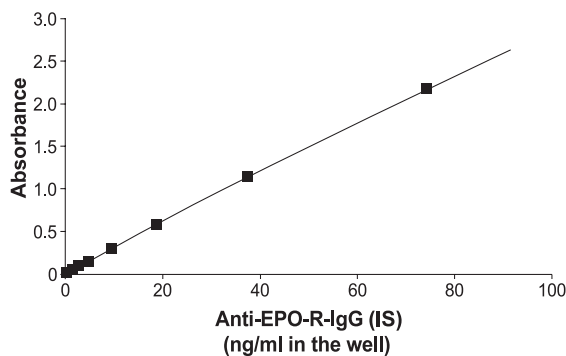


Fig. 2. Calibration curve of the anti-EPO double antigen bridging assay. Polyclonal anti-EPO Abs from rabbits purified by immunoadsorption were used as standard material.

of anti-EPO Abs measured 13 times over a period of 2 months was 12.6%. A hook effect was not observed when high concentrations of the reference material were used in the assay.

3.2. Cut-off determination

Serum samples ($n=159$) randomly chosen from different hospitals and blood banks, and not considered to contain anti-EPO Abs, were analyzed by the standard assay as a representative collection of negative samples. Relative to the reference material, the 90th percentile of these samples was determined at

≤ 5 ng/ml, the 97.5th percentile was at 8 ng/ml, and the 99th percentile was at 10 ng/ml (see Fig. 3). Repetition of the cut-off determination using a collection of 200 negative sera from dialysis patients revealed similar values. Upon routine analysis of sera, three samples of reference material covering the assay concentration range were always included in the assays, and the recoveries observed were all within $\pm 20\%$ of the expected value. In order to identify as many anti-EPO Ab-positive samples as possible, all samples which revealed values above 1 ng/ml in the first round of measurement were analyzed again, employing different amounts of serum and performing displacement reactions with rhEPO in order to differentiate between nonspecific and specific signals (see next two chapters).

3.3. Analysis of anti-EPO Ab-positive samples

All of the 20 samples previously identified as containing anti-EPO Abs (see Methods) revealed clear positive results with this new double antigen bridging ELISA. The values measured from these positive samples were all well above the 99% percentile cut-off of 10 ng/ml calibration material equivalents, ranging from about 40 up to 30,000 ng/ml.

In order to analyze the influence of serum erythropoietin concentrations on the results obtained

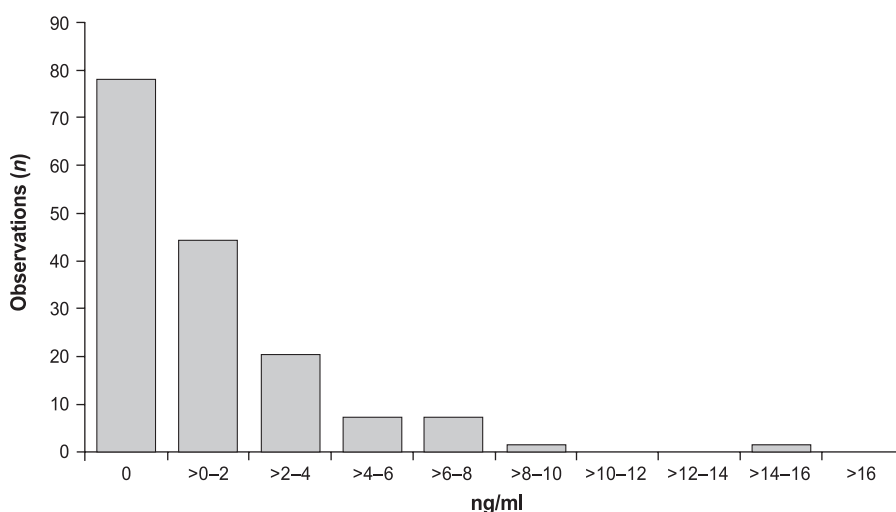


Fig. 3. Distribution of the values obtained with the anti-EPO Ab-negative reference group. The reference sera were selected from healthy persons, SLE, RA, and SS patients. Reference limits were calculated at 5 ng/ml for the 90th percentile and 15.5 ng/ml for the 99.9th percentile.

with this assay, displacement curves were determined by adding increasing concentrations of rhEPO to assays with control material and three anti-EPO Ab-positive samples. The results obtained are illustrated in Fig. 4. All curves follow a similar pattern, with 50% displacement observed in the range of rhEPO concentrations from 2–20 ng/ml. Inasmuch as serum erythropoietin concentrations observed during rhEPO therapy are usually in the range 0.1–100 ng/ml (Allon et al., 2002), it can be concluded that the results obtained with this anti-EPO assay are not greatly influenced by serum EPO levels. Indeed, only at the highest concentration (100 ng/ml) would an approximate 50% decrease in absorption be observed (Fig. 4). Inasmuch as steady state levels are usually well below 10 ng/ml (Allon et al., 2002), they would not have much impact on the concentration of anti-EPO Abs measured with this assay. On the other hand, it seems obvious that the presence of anti-EPO Abs will have a profound influence on the EPO values obtained with immunological tests using these sera (a considerable underestimation is likely), and this has already been observed (Tacey et al., 2003).

No inhibition or interference occurred in the assays (data not shown), as indicated by the complete recovery of anti-EPO Abs in the negative sera in experiments involving mixing of anti-EPO Ab-negative and -positive sera.

Inasmuch as this new test is suitable for the relative quantification of anti-EPO Abs, it was used for a follow up analysis of the anti-EPO Ab concentrations

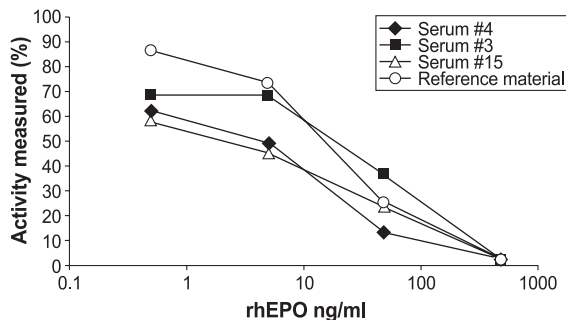


Fig. 4. Displacement of absorbance by increasing concentrations of unlabelled rhEPO in the anti-EPO ELISA with reference material and sera of three patients. Anti-EPO concentrations were as follows (ng/ml in the wells): serum #3—6.3; #4—19; #15—60; reference material—10.

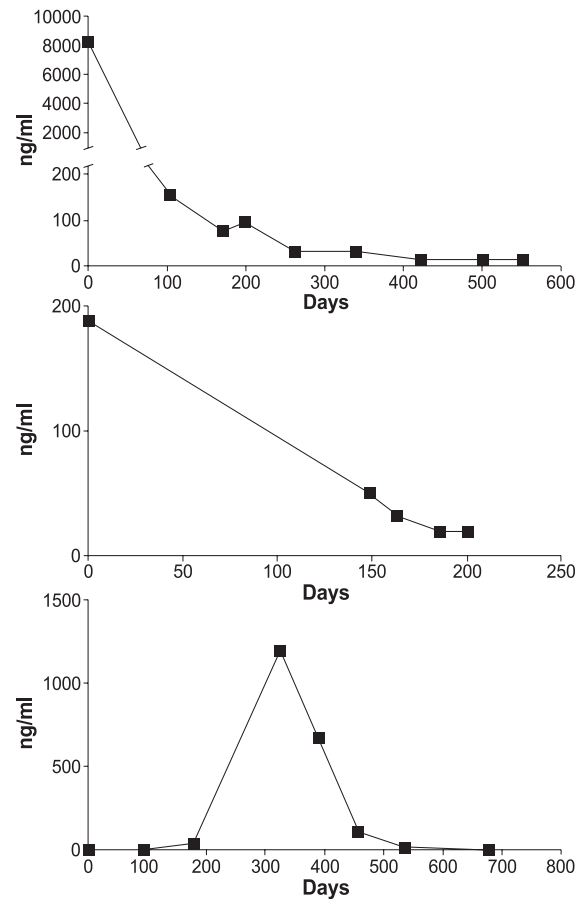


Fig. 5. Illustration of the decrease of anti-EPO Ab concentrations in three patients after discontinuation of rhEPO treatment.

of several PRCA patients whose rhEPO treatment had been discontinued at the time of diagnosis. As can be seen from Fig. 5, a continuous decrease of the anti-EPO Ab concentrations was observed, albeit with different kinetics, for the three patients studied.

An important practical question for the analysis of anti-EPO Abs is their stability under storage conditions. To study whether these antibodies remained reactive when stored for an extended period of time, aliquots of positive serum samples were stored under different conditions (see Table 1). The reactivity of anti-EPO Abs did not decline after storage at $-20\text{ }^{\circ}\text{C}$ for 5 months, at $4\text{ }^{\circ}\text{C}$ or room temperature for 3 days, or at $37\text{ }^{\circ}\text{C}$ for 24 h. Several freeze/thaw cycles of undiluted anti-EPO positive sera had no influence on the quantitative results obtained.

3.4. Analysis of other pathological sera

There have been several reports in the literature that patients with autoimmune diseases (e.g., SLE) have autoantibodies to EPO (Tzioufas et al., 1997; Voulgarelis et al., 2000; Schett et al., 2001). Moreover, in one study, a high percentage (67%) of rhEPO-treated dialysis patients tested positive for the presence of anti-EPO Abs with low affinity for the antigen (Castelli et al., 2000), and in another study, HIV patients with anemia had circulating autoantibodies against EPO (Sipsas et al., 1999). Inasmuch as all of these studies used ELISAs without displacement steps, which are prone to high rates of false positive results, we also analyzed sera of patients with these diseases. The results revealed that, with the possible exception of one SLE patient (see below), all sera from SLE, RA, Sjögren's syndrome, and dialysis patients revealed similar results as the negative controls.

Among the SLE patients, one sample was observed with a value above the cut-off (12 ng/ml). Inasmuch as this was an interesting case representing a sample in a "grey area" for determinations, it was further characterized in order to find out whether it was truly positive for anti-EPO Abs. The serum was serially diluted, and the results were compared with those of two similarly diluted positive samples. The positive samples increased linearly with increasing serum concentrations, whereas this SLE sample reached a plateau at a low level (data not shown). Therefore, this indicated for this sample that some nonspecific reaction was the reason for the value slightly above the cut-off

Table 1
Stability of anti-EPO Abs under different storage conditions (% recovery of the starting concentration)

Starting concentration		Patient No. 1 (10.4 µg/ml)	Patient No. 2 (1.7 µg/ml)
Storage conditions		% recovered	
Temperature	Duration		
–20 °C	5 months	100	100
4 °C	3 days	93	106
RT	3 days	89	106
37 °C	24 h	91	106

RT—room temperature

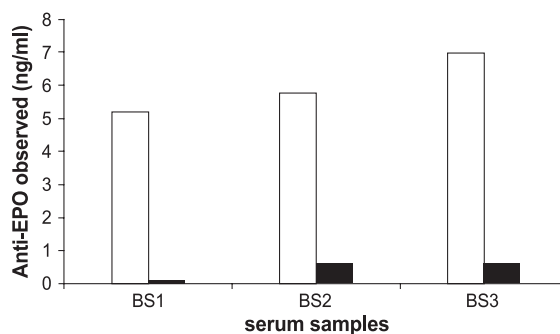


Fig. 6. Displacement of the anti-EPO Ab response by rhEPO in serum samples from three PRCA patients whose anti-EPO Ab measurements were near the cut-off value. The experiment demonstrates that they did contain anti-EPO Abs (30 ng/ml rhEPO in the assay). (open bar=–rhEPO; solid bar=+rhEPO, 30 ng/ml in the assay.)

level. This sample was also tested using RIPA and with an ELISA with displacement, and both results were negative.

A further much more stringent method to verify the presence of anti-EPO Abs in low titer sera near the cut-off is a competition experiment as illustrated in Fig. 6. In the presence of EPO, the absorbance values obtained in the assay will decrease to background levels because the binding of EPO-DIG is inhibited by the surplus of unlabelled EPO in the assay mixture.

4. Discussion

A suitable laboratory test for the analysis of anti-EPO Ab in human sera should be sensitive, specific, and provide a quantitative assessment of anti-EPO Ab concentrations. Moreover, it should be convenient to use for the analysis of large numbers of serum samples. None of the assays reported to date meet all of these criteria. However, the double antigen bridging ELISA described above meets these criteria to a large extent.

The test features the bridging of two separate rhEPO-molecules by anti-EPO Abs, and this principle is the basis for the high specificity observed with this ELISA. The range of the calibration curve illustrated in Fig. 2 was chosen with the aim of achieving as high a sensitivity as possible to allow the detection of low-level anti-EPO Ab-positive samples. In order to realize the specificity and

sensitivity of this assay, it is very important to follow the optimized test protocol as closely as possible, for example, the mixture of the serum and the rhEPO-DIG should be added to the wells without delay.

The bridging ELISA is convenient and can be used to quantify anti-EPO Ab concentrations relative to the reference material. Using this assay, the kinetics of anti-EPO Ab concentrations were measured as a follow-up to anti-EPO Ab detection in PRCA patients. A steep decrease in Ab concentration was found in one PRCA patient after stopping rhEPO therapy, while a much slower decrease was observed with the other two subjects (Fig. 5). A slow decrease in anti-EPO Abs has also been reported by Casadevall et al. (2002). These measurements provide important information on the differing rates of decrease in anti-EPO Ab concentrations in individual patients. Moreover, the anti-EPO Ab background level for patients can be determined due to the high sensitivity of this test. Hence, an increase in the anti-EPO Ab concentrations, if it occurred, could be easily identified during patient monitoring.

Very recently, Swanson et al. (2004) described an anti-EPO ELISA with a sensitivity of 780 ng/ml, using affinity-purified polyclonal anti-rhEPO antibodies from rabbits as reference material. Assuming that the two reference preparations used were of equal purity and similar properties (e.g., affinity), our ELISA described above was at least 50 times more sensitive than the one described by Swanson et al. (2004). It is therefore reasonable to assume that the two sera, which were reported as negative in their ELISA, would have tested positive in ours. How the sensitivity of our ELISA compares to the RIPA described by Casadevall et al. (2002) has to be determined. Although Rossert et al. (2004) claim that the RIPA is slightly more sensitive than our ELISA (Casadevall, personal communication), this has not been demonstrated unequivocally to our knowledge. A head to head comparison of these two assays together with the BIAcore assay (Mason et al., 2003) is now under way in several laboratories.

The rabbit polyclonal anti-EPO IgG used in this investigation will serve as a long-term reference material for this ELISA. Anti-EPO antibodies of human origin could, of course, behave differently in this ELISA, e.g., due to different affinities. However,

as demonstrated in Fig. 4, the anti-EPO IgG from rabbits and the three human sera displayed IC_{50} values in the same concentration range, suggesting similar properties.

Another important feature of a diagnostic test is high specificity, which would keep the risk of false positive results to a minimum. This is particularly important if a large number of patients have to be tested and only a few positives are to be expected, as is the case with the low prevalence of anti-EPO Abs. Moreover, the results obtained clearly demonstrate that the earlier reports of a high frequency of anti-EPO Abs in patients with renal failure and treated with rhEPO have to be regarded with much caution. Castelli et al. (2000) reported that 67% of renal failure patients treated with rhEPO were positive for anti-EPO Abs, using their assay. Casadevall et al. (2002) assumed that these were low-affinity antibodies that did not neutralize erythropoietin. Sipsas et al. (1999) observed circulating anti-EPO autoantibodies in 48% of patients infected with immunodeficiency virus type 1 (HIV-1). In addition, Voulgarelis et al. (2000) reported anti-EPO Abs in 21 of 100 patients with anemia of chronic disease (ACD) or with high European Consensus Lupus Activity Measure (ECLAM) scores. Inasmuch as all these data were obtained using ELISAs performed without confirmation of a displacement step and are therefore prone to nonspecific reactions, they are very likely to be false positive results. Indeed, a high incidence of anti-EPO Abs would be very surprising in any pathological state except for PRCA, and it would contradict earlier reports that the occurrence of anti-EPO Abs was a rare event (Casadevall, 2002, 2003). The high incidence of false positives in patients with autoimmune diseases such as RA, SLE, and Sjögren's syndrome may be due to the presence of rheumatoid factor (RF) type Igs in the sera. Inasmuch as these assays are based on the detection of bound Ig, any nonspecific binding of Ig to the wall will give a signal. Moreover, RF factors are known to increase binding due to their inherent protein characteristics (Johnson and Faulk, 1976). The measurement of sera from SLE, RA, Sjögren's syndrome, and dialysis patients with the bridging ELISA described here failed to confirm the high incidence of anti-EPO Ab-positive samples reported in previous cases (Tzioufas et al., 1997; Sipsas et al., 1999; Castelli et al., 2000; Voulgarelis et al., 2000;

Schett et al., 2001). Tacey et al. (2003) have also recently confirmed that sera from patients with chronic diseases such as RA and SLE did not contain anti-EPO Abs when analyzed with a RIPA. Finally, these findings demonstrate that the addition of a competitive displacement step adds specificity to an ELISA.

In the meantime, another 1500 serum samples have been analyzed with this bridging ELISA since the development of the test, and in general, the sera containing anti-EPO Abs had values that were very clearly above the cut-off, indicating the suitability of this assay for screening purposes. In the few cases where values near the cutoff were obtained, competition experiments using rhEPO could clearly distinguish whether specific binding to EPO or nonspecific binding to the well was responsible for the signal observed. In summary, we conclude that the double antigen sandwich ELISA described here is suitable for the detection and quantification of anti-EPO Abs in human sera.

Acknowledgements

This work was supported by grants from the Humboldt University, Berlin, grant No. 2003-415 and from Roche Diagnostics.

References

- Allon, M., Kleinman, K., Walczyk, M., Kaupke, C., Messer-Mann, L., Olson, K., Heatherington, A.C., Maroni, B.J., 2002. Pharmacokinetics and pharmacodynamics of darbepoetin alfa and epoetin in patients undergoing dialysis. *Clin. Pharmacol. Ther.* 72, 546.
- Besarab, A., Erslev, A.J., 1997. EPO in pathogenesis and treatment of the anemia of chronic renal failure. *Kidney Int.* 51, 622.
- Bunn, H.F., 2002. Drug-induced autoimmune red-cell aplasia. *N. Engl. J. Med.* 346, 522.
- Casadevall, N., 2002. Antibodies against rHuEPO: native and recombinant. *Nephrol. Dial. Transplant.* 17 (Suppl. 5), 42.
- Casadevall, N., 2003. Pure red cell aplasia and anti-erythropoietin antibodies in patients treated with epoetin. *Nephrol. Dial. Transplant.* 18 (Suppl. 8), viii37.
- Casadevall, N., Dupuy, E., Molho-Sabatier, P., Tobelem, G., Varet, B., Mayeux, P., 1996. Autoantibodies against erythropoietin in a patient with pure red-cell aplasia. *N. Engl. J. Med.* 334, 630.
- Casadevall, N., Nataf, J., Viron, B., Kolta, A., Kiladjian, J.J., Martin-Dupont, P., Michaud, P., Papo, T., Ugo, V., Teysandier, I., Varet, B., Mayeux, P., 2002. Pure red-cell aplasia and antierythropoietin antibodies in patients treated with recombinant erythropoietin. *N. Engl. J. Med.* 346, 469.
- Castelli, G., Famularo, A., Semino, C., Machi, A.M., Ceci, A., Cannella, G., Melioli, G., 2000. Detection of anti-erythropoietin antibodies in haemodialysis patients treated with recombinant human-erythropoietin. *Pharmacol. Res.* 41, 313.
- Dunn, C.I., 1996. Epoetin beta. A review of its pharmacological properties and clinical use in the management of anemia associated with chronic renal failure. *Drugs* 51, 299.
- Gershon, S.K., Luksenburg, H., Cote, T.R., Braun, M.M., 2002. Pure red-cell aplasia and recombinant erythropoietin. *N. Engl. J. Med.* 346, 1584.
- Haselbeck, A., Hoesel, W., 1999. Labeling and detection of proteins and glycoproteins. In: Kessler, C. (Ed.), *Nonradiative Labeling and Detection of Biomolecules*. Springer Verlag, Germany, p. 94.
- Hermeling, S., Schellekens, H., Crommelin, D.J.A., Jiskoot, W., 2003. Micelle-associated protein in epoetin formulations: a risk factor for immunogenicity? *Pharmacol. Res.* 20, 1903.
- Johnson, P.M., Faulk, W.P., 1976. Rheumatoid factor: its nature, specificity and production in rheumatoid arthritis. *Clin. Immunol. Immunopathol.* 6, 414.
- Kientsch-Engel, R., Hallermayer, K., Dessauer, A., 1989. Methods for measuring erythropoietin and erythropoietin antibodies using ELISA technique. In: Berlyne, G.M., Giovannetti, S. (Eds.), *Contributions to Nephrology*, vol.76, p. 100.
- Mason, S., La, S., Mytych, D., Swanson, S.J., Ferbas, J., 2003. Validation of the BIAcore 3000 platform for detection of antibodies against erythropoietic agents in human serum samples. *Curr. Med. Res. Opin.* 19, 651.
- Peter, J., Unverzagt, C., Lenz, H., Hoesel, W., 1999. Purification of prostate-specific antigen from human serum by indirect immunosorption and elution with a haptin. *Anal. Biochem.* 273, 98.
- Rosert, J., Casadevall, N., Eckardt, K.U., 2004. Anti-erythropoietin antibodies and pure red cell aplasia. *J. Am. Soc. Nephrol.* 15, 398.
- Schett, G., Firbas, U., Füreder, W., Hiesberger, H., Winkler, S., Wachauer, D., Köller, M., Kapiotis, S., Smolen, J., 2001. Decreased serum erythropoietin and its relation to anti-erythropoietin antibodies in anemia of systemic lupus erythematosus. *Rheumatology* 40, 424.
- Sipsas, N.V., Kokori, S.I., Ioannidis, J.P., Kyriaki, D., Tzioufas, A.G., Kordosis, T., 1999. Circulating autoantibodies to erythropoietin are associated with human immunodeficiency virus type 1-related anemia. *J. Infect. Dis.* 180, 2044.
- Swanson, S.J., 2003. New technologies for the detection of antibodies to therapeutic proteins. In: Brown, F., Mire-Sluis, A.R. (Eds.), *Immunogenicity of Therapeutic Biological Products*, Dev. Biol., vol. 112. Karger, Basel, p. 127.
- Swanson, S.J., Ferbas, J., Mayeux, P., Casadevall, N., 2004. Evaluation of methods to detect and characterize antibodies against recombinant human erythropoietin. *Nephron Clin. Pract.* 96, 88.
- Tacey, R., Greway, A., Smiell, J., Power, D., Kromminga, A., Daha, M., Casadevall, N., Kelley, M., 2003. The detection of anti-erythropoietin antibodies in human serum and plasma: Part I.

- Validation of the protocol for a radioimmunoprecipitation assay. *J. Immunol. Methods* 283, 317.
- Tzioufas, A.G., Kokori, S.I., Petrovas, C.I., Moutsopoulos, H.M., 1997. Autoantibodies to human erythropoietin in patients with systemic lupus erythematosus: correlation with anemia. *Arthritis Rheum.* 40, 2212.
- Urra, J.M., de la Torre, M., Alcazar, R., Peces, R., 1997. Rapid method for detection of anti-recombinant human erythropoietin antibodies as a new form of erythropoietin resistance. *Clin. Chem.* 43, 848.
- Voulgarelis, M., Kokori, S.I., Ioannidis, J.P., Tzioufas, A.G., Kyriaki, D., Moutsopoulos, H.M., 2000. Anemia in systemic lupus erythematosus: aetiological profile and the role of erythropoietin. *Ann. Rheum. Dis.* 59, 217.
- Weber, G., Gross, J., Kromminga, A., Loew, H.H., Eckardt, K.U., 2002. Allergic skin and systemic reactions in a patient with pure red cell aplasia and anti-erythropoietin antibodies challenged with different epoetins. *J. Am. Soc. Nephrol.* 13, 2381.