

Research paper

# Detection of anti-EPO antibodies in human sera by a bridging ELISA is much more sensitive when coating biotinylated rhEPO to streptavidin rather than using direct coating of rhEPO

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## Abstract

Sensitive and efficient methods for detecting anti-erythropoietin (anti-EPO) antibodies are needed for analysis and, above all, for large scale screening of human serum samples. ELISA is an attractive alternative to labor-intensive radioimmuno-precipitation assays but apparently conflicting reports question its sensitivity. We sought to resolve this issue by directly comparing different reported ELISA approaches to determine whether rhEPO-coating methods affect detection of anti-EPO antibodies. Investigators reporting low sensitivity had used ELISAs in which rhEPO was directly coated to microtiter plates while the high sensitivity ELISA used plate-bound streptavidin to bind biotinylated rhEPO. Using anti-EPO positive human sera, our results confirmed a large (100- to 300-fold) difference in sensitivity between the ELISAs and suggested that the inferiority of the low sensitivity ELISA was caused by the direct coating of rhEPO which may disrupt epitopes by masking recognition sites or introducing conformational changes. Thus, a bridging ELISA can be an appropriate and effective system for antibody analysis and screening of human sera with high sensitivity and specificity but only if performed with streptavidin binding of biotinylated antigen. This finding may also be more generally applicable to the detection of antibodies against other protein antigens.

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## 1. Introduction

Sensitive and reproducible assays for the measurement of anti-erythropoietin (anti-EPO) antibodies are important for the diagnosis of EPO-induced pure red cell aplasia (Casadevall et al., 2004). In early studies, screening of human serum for anti-EPO antibodies was primarily performed using a radioimmunoprecipitation assay (RIPA) (Casadevall et al., 1996, 2002; Tacey et al., 2003). However, as RIPA requires the use

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*Abbreviations:* DIG, digoxigenin; LLD, lower limit of detection; LLQ, lower limit of quantification; rhEPO, recombinant human erythropoietin; RIPA, radio-immunoprecipitation assay; RT, room temperature; MTP, microtiter plate; UNB, universal buffer.

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of radioactive material and is laborious to perform, our laboratory investigated other alternatives for the analysis of human sera. This led to the development and extensive characterization of a double antigen-bridging ELISA demonstrating advantages such as high specificity and good practicality over other immunoassay formats for the analysis of anti EPO antibodies (Hoesel et al., 2004). The double antigen-bridging refers to the simultaneous binding of anti-EPO antibodies to two forms of recombinant human EPO (rhEPO) antigen—one biotinylated form immobilized to streptavidin-coated microtiter plates (MTPs) and a second form labeled with digoxigenin (DIG) to permit colorimetric quantitation via anti-DIG antibodies coupled to horseradish peroxidase (HRP). Using immunopurified rabbit polyclonal anti-rhEPO IgG as reference, this double antigen-bridging ELISA also showed good sensitivity with an estimated lower limit of detection (LLD) of 1 ng/ml. It proved to be very suitable for the analysis of more than 5000 human sera for the presence and relative quantification of anti EPO antibodies in rhEPO treated patients (Hoesel, Gross, et al.; in preparation).

Despite these findings, there remains some disagreement over the relative sensitivity of an ELISA compared to other screening assays, such as RIPA, for effective screening of human serum (Casadevall et al., 2004; Swanson et al., 2004; Swanson, 2003; Thorpe and Swanson, 2005; Locatelli et al., 2004). However, this is largely based on a report of an anti-EPO bridging ELISA which used non-modified rhEPO for immobilisation to MTPs and biotinylated rhEPO with a streptavidin–HRP conjugate for detection (Swanson et al., 2004). This ELISA showed a sensitivity of only 780 ng/ml using affinity-purified rabbit polyclonal anti-rhEPO antibodies as reference material and failed to detect anti-EPO antibodies in two sera from patients which were found by RIPA to contain comparatively high quantities of anti-EPO (6.43 and 4.29 µg/ml, respectively). Clearly, the poor LLD of this ELISA contrasts with the high sensitivity shown in our report (Hoesel et al., 2004) and has led many investigators to question the appropriateness of double antigen-bridging ELISAs for analysis of anti-EPO antibodies of rhEPO-induced pure red cell aplasia patients (Casadevall et al., 2004; Swanson et al., 2004; Swanson, 2003; Thorpe and Swanson, 2005; Locatelli et al., 2004).

To resolve this discrepancy, we sought to identify what might be contributing to the wide variation in sensitivities of the two double antigen-bridging ELISAs. The main difference is the method of rhEPO immobilization to MTPs—direct absorption of non-modified

rhEPO (Swanson et al., 2004) versus the use of streptavidin to capture biotinylated rhEPO (Hoesel et al., 2004). Thus, we directly studied the effect of the coating procedure on LLD using the same secondary detection method to quantify rabbit anti-EPO IgG (calibrator) and anti-EPO positive human sera. Our results suggest that the direct immobilization of rhEPO to the MTP surface is very likely to be the main reason for the low sensitivity observed by Swanson et al. (2004) for the detection of anti EPO antibodies in human sera.

## 2. Materials and methods

### 2.1. Materials

MTPs precoated with streptavidin were obtained from Microcoat GmbH (Bernried, Germany). Rabbit anti-EPO polyclonal antibody (immunosorbed), rhEPO, anti-DIG-HRP conjugate, ABTS (2,2'-Azino-di-[3-ethylbenzthiazoline sulfonate(6)] diammonium salt) and all reagents for the incubation buffers of the ELISAs were obtained from F. Hoffman-La Roche Diagnostics GmbH (Penzberg, Germany). All other reagents were from Merck, GmbH (Germany), if not indicated otherwise.

### 2.2. Serum samples

Sera from rhEPO-treated patients who were diagnosed as having anti-EPO antibodies were used. Control serum samples (negative for anti-EPO antibody) were collected from blood banks, dialysis centres and hospitals by Hoffman-La Roche Diagnostics GmbH.

### 2.3. Anti-EPO ELISA using streptavidin and biotinylated rhEPO for coating (streptavidin/biotin-coated ELISA)

The basic protocol for anti-EPO double antigen-bridging ELISA was carried out as previously described (Hoesel et al., 2004). Briefly, a mixture of biotinylated rhEPO (linked via carbohydrate groups) (0.75 µg/ml) and biotinylated rhEPO (linked via amino groups) (0.5 µg/ml) in universal buffer (UNB, Roche) was incubated in a streptavidin-coated MTP at a final volume of 125 µl/well for 30 min at room temperature (RT). The wells were then washed three times with washing buffer (WB) consisting of potassium phosphate buffer (40 mM, pH 7.4) and Tween 20 (0.1%). Human control serum diluted 1 in 5 (v/v) with UNB alone or

with reference antibody preparation (rabbit polyclonal anti-EPO antibody) were mixed with an equal volume of DIG-labeled rhEPO (40 ng/ml in UNB). This solution (100  $\mu$ l) was added to wells without delay and incubated for 2 h (shaking at 300 rpm). After washing three times with WB, 100  $\mu$ l of anti-DIG Fab fragments-HRP conjugate (75 mU/ml) freshly prepared in UNB were added to each well and incubated for 1 h (shaking at 300 rpm). After washing (three times with WB), 100  $\mu$ l ABTS solution (1 mg/ml ABTS in ABTS buffer) were added to each well and incubated for 30–60 min. Absorbance values were measured at 405 nm and at 492 nm for reference. Titration of immunopurified anti-EPO IgG from rabbits was used to calculate calibration curves using 4-parameter regression. All incubations were performed at RT with MTPs sealed by plastic covers. The total time required to complete the protocol was approximately 5 h. Assays run overnight were conducted similarly, except for incubation of samples with rhEPO–DIG which was performed as the overnight step at 37 °C.

#### 2.4. Anti-EPO ELISAs using direct coating of rhEPO (directly coated ELISA)

Directly coated ELISAs were performed either as described by (Swanson et al., 2004) or as follows. MTPs were coated with rhEPO (Roche) diluted in phosphate buffer (50 mM, pH 7.2,) or in sodium carbonate/bicarbonate buffer (0.1 M, pH 9.5) to a concentration of 500 to 5000 ng/ml overnight at 4 °C. Thereafter, the rhEPO solution was replaced by blocking reagent (20 g/l sucrose, 3 g/l BSA, 9 g/l NaCl and 2 mM K<sub>3</sub>PO<sub>4</sub>, pH 7.2, 0.05% Tween 20) for 1 h and washed 3 times with WB. All further steps to complete the directly coated ELISA were identical to the streptavidin/biotin-coated ELISA described above.

#### 2.5. Determination of 50% inhibitory concentration (IC<sub>50</sub>)

The IC<sub>50</sub> of unlabeled rhEPO was determined by incubating anti-EPO antibodies with increasing concentrations (0.5 to 300 ng/ml) of unlabeled rhEPO. Unlabeled rhEPO was added to wells together with DIG-labeled rhEPO. The IC<sub>50</sub> of rhEPO was calculated by nonlinear regression analysis of the experimental data using Eq. (1).

$$\text{antiEPOAb}_{\text{bound}} = \frac{\text{antiEPOAb}_{\text{max}} \times \text{IC}_{50}}{\text{rhEPO} + \text{IC}_{50}} \quad (1)$$

AntiEPOAb<sub>max</sub> denotes the bound anti-EPO antibody in the absence of rhEPO.

### 3. Results

#### 3.1. Calibration curves

The coating conditions used for the streptavidin/biotin-coated ELISA (Hoesel et al., 2004) and the direct-coated ELISA (Swanson et al., 2004) were as described. For the latter, additional coating conditions were evaluated by testing 0.5, 1, 2 or 5  $\mu$ g/ml rhEPO and by comparing sodium carbonate buffer (pH 9.5) and potassium phosphate buffer (pH 7.2). A higher absorbance was achieved with sodium carbonate buffer compared to potassium phosphate buffer (approximately 20%), but no differences were observed for the various concentrations of rhEPO (data not shown). Therefore, all subsequent directly coated ELISAs were done using 1  $\mu$ g/ml rhEPO in sodium carbonate buffer (pH 9.5).

In a first series of experiments we compared the calibration curves obtained by the streptavidin/biotin-coated ELISA and the two directly coated ELISAs developed here and by Swanson et al. (2004) using the rabbit immunopurified anti-EPO IgG as calibrator. All ELISA conditions gave consistent calibration curves within the same sensitivity range (see Fig. 1).

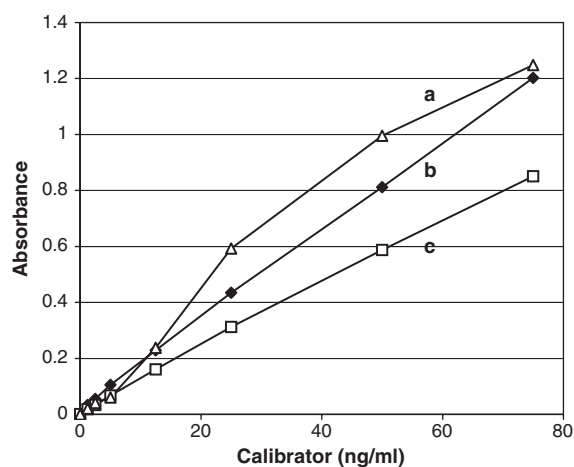


Fig. 1. Comparison of the standard curves obtained with streptavidin/biotin-coated ELISA and directly coated ELISAs. (a) Directly coated ELISA according to Swanson et al. (2004). (b) Streptavidin/biotin-coated ELISA. (c) Directly coated ELISA with coating in carbonate buffer pH 9.5, but otherwise identical to (b). Polyclonal anti-EPO antibodies from rabbits were used as reference material. A representative example of the calibration curve of each test is shown.

Table 1

Comparison of anti-EPO antibody levels (ng/ml) of human sera when assayed by streptavidin/biotin coated and directly coated ELISAs using different incubation conditions

Serum no.	Streptavidin/ biotin-coated ELISA (RT, 2 h)	Directly coated ELISA (RT, 2 h)	Directly coated ELISA (37 °C, overnight)
1	1.1	0	3.6
2	3.2	0	1.4
3	6.7	0	2.5
4	8.4	0	5.3
5	18.2	0	9.7
6	22.8	0	2.5
7	380	0	6.4
8	391	0	2.5
9	880	10.0	25.9
10	993	3.2	14.2
11	2182	9.5	28.2
12	2840	12.4	41.1
13	4405	24.4	53.5
14	5479	25.0	87.0
15	7005	27.9	72.2

### 3.2. Comparison of anti-EPO antibody levels in human sera detected by streptavidin/biotin-coated and directly coated ELISAs

In a further series we directly compared the sensitivities of the directly coated ELISAs and the streptavidin/biotin-coated ELISA using human sera. Several samples were tested, each known to contain a range of low to high titers of anti-EPO antibody but all within the sensitivity range of the ELISAs as determined in Fig. 1. Surprisingly, the directly coated ELISAs failed to detect anti-EPO antibody in the low titer sera when performed at RT using the conditions described in Materials and methods. In contrast, these sera were clearly positive in the streptavidin/biotin-ELISA. High titer sera were positive by all ELISAs, although the streptavidin/biotin-coated ELISA always generated 100 to 300 times higher concentration values than the directly coated ELISA (see Table 1).

A possible cause for these discrepancies may have been variation in efficiencies of anti-EPO antibody binding to MTPs in the 2 h incubation time at RT. To test this, all ELISAs were repeated with overnight incubations of samples and rhEPO-DIG at 37 °C—conditions which have been found optimal in preliminary experiments (data not shown). Table 1 lists all results obtained with the two assays for the low and high titer sera, and compares the two incubation conditions used (RT and 37 °C; 2 h and overnight). The overnight incubations resulted in increased detection of anti-EPO antibody in the directly coated ELISAs, yet all high titer sera still

generated lower values than streptavidin/biotin-coated ELISAs performed with sample incubations for 2 h at RT.

The large differences in sensitivities observed for detection of rabbit anti-EPO IgG compared with human serum anti-EPO antibodies by the two types of ELISAs was striking. It is possible that serum components contributed to the low sensitivity of the directly coated ELISAs by interfering with the recognition of EPO by anti-EPO antibodies, perhaps by limiting their binding or changing their affinities. At first we measured an anti-EPO negative serum spiked with increasing concentrations of the calibrator (rabbit polyclonal anti-EPO antibodies). For each ELISA, recovery of the calibrator appeared unaffected by serum components (see Fig. 2). This also held true when the calibrator was diluted with UNB-buffer (data not shown). Next we diluted anti EPO positive serum with negative serum or with UNB buffer. Identical recovery of the anti EPO positive human serum was observed upon dilution in UBS or in negative human serum for the streptavidin/biotin-coated ELISA as well as the directly coated ELISA (see Fig. 3). Therefore components of the serum matrices seem not to be responsible for the low sensitivity of the directly coated ELISA for human anti-EPO antibody detection.

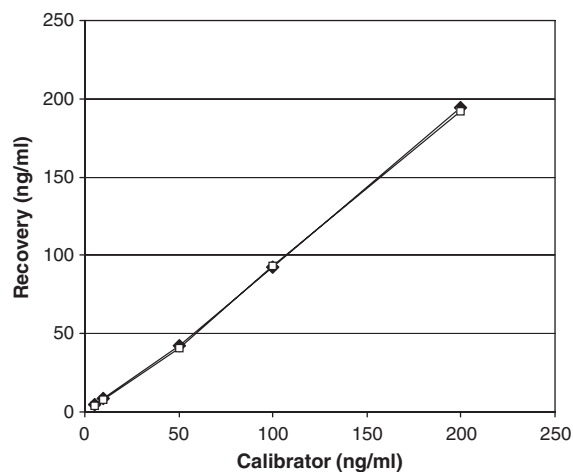


Fig. 2. Recovery of calibrator (polyclonal anti EPO Ab from rabbits) using a streptavidin/biotin-coated ELISA and a directly coated ELISA. Calibrator (600 ng/ml) was diluted with a negative serum to concentrations of 5, 10, 50, 100, 200 ng/ml and measured using a streptavidin/biotin-coated ELISA (filled squares) and a directly coated ELISA (open squares). The CV for the streptavidin/biotin-coated ELISA was 12% for recovery at the level of 5 and 10 ng/ml and 2% at the level of 50, 100 and 200. The CV for the directly coated ELISA was 25% for recovery at the level of 5 and 10 ng/ml and 2% at the level of 50, 100 and 200.

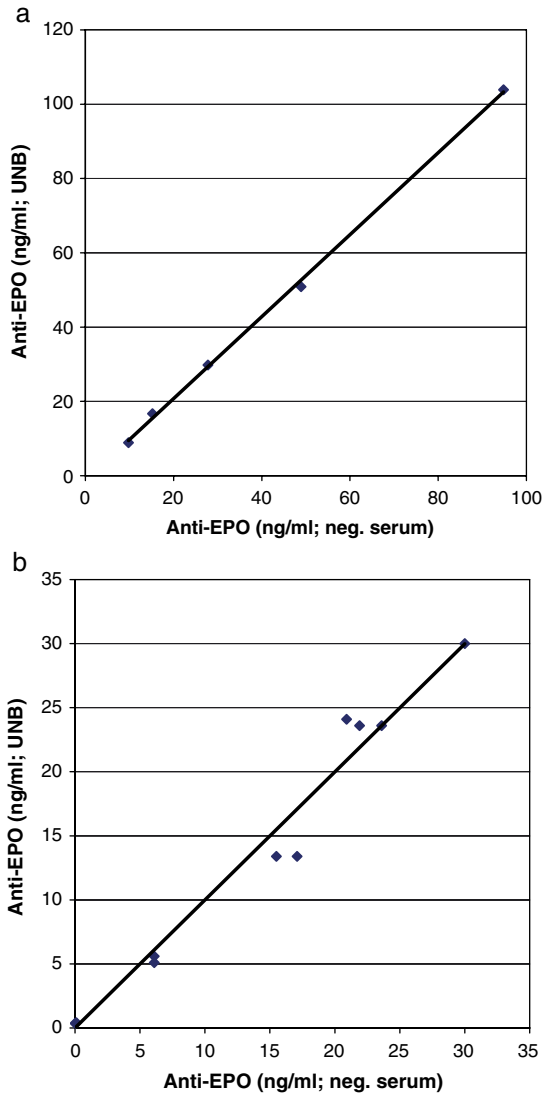


Fig. 3. Recovery of positive serum using the streptavidin/biotin-coated ELISA and directly coated ELISA procedures. Positive serum was diluted with a negative serum (abscissa) or with UNB buffer (ordinate) to different antibody concentrations and was measured using the streptavidin/biotin-coated ELISA (a) and the directly coated ELISA (b). The positive serum used for this comparison showed an activity of 4500 ng/ml and 8364 ng/ml in the streptavidin/biotin-coated ELISA. The corresponding activities in the directly coated ELISA were 30 ng/ml and 23.6 ng/ml, respectively. A close correlation was found for both tests: (a)  $y=1.10x+0.5$ ;  $R^2=0.99$ ; (b)  $y=1.0-0.5$ ;  $R^2=0.97$ .

### 3.3. $IC_{50}$ values of calibrator and human sera in streptavidin/biotin-coated and directly coated ELISAs

To analyze whether variations in anti-rhEPO antibody affinities in calibrator and human sera contribute to the sensitivity differences observed with

both ELISA designs, we studied antibody inhibition by unlabeled rhEPO (Fig. 4).  $IC_{50}$  values of rhEPO were calculated by nonlinear regression analysis. For the calibrator, streptavidin/biotin-coated and directly coated ELISAs displayed  $IC_{50}$  values of 1.6 nM and 3.9 nM, respectively. For two positive sera, the  $IC_{50}$  values were consistently lower for both the streptavidin/biotin-coated ELISA (0.22 nM and 0.29 nM) and the directly coated ELISA (0.05 nM and 0.3 nM). Hence, the affinity of the calibrator for rhEPO is 5–10 times lower than that of the anti-EPO antibodies of the two human sera, but there was not a great difference between the two ELISA types regarding the observed  $IC_{50}$  values.

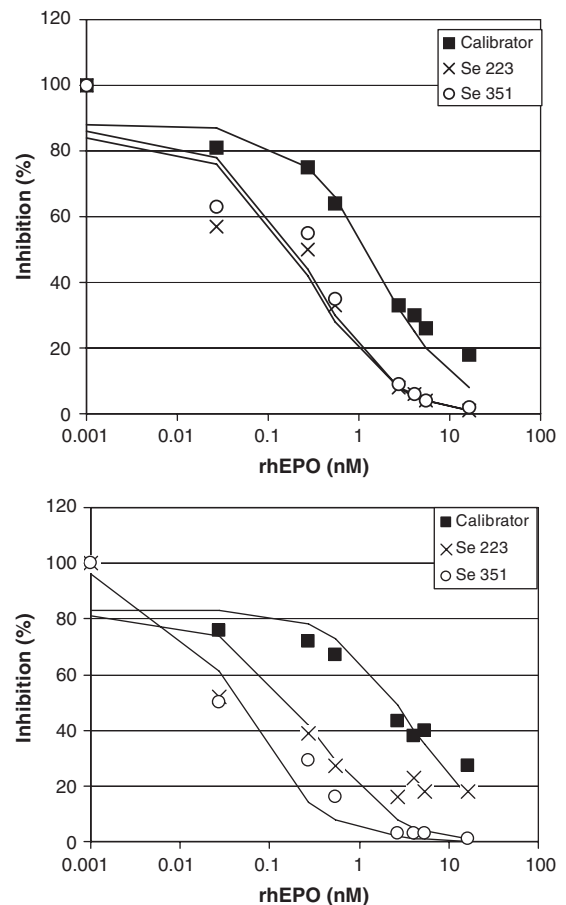


Fig. 4. Binding inhibition of calibrator and human anti-rhEPO antibodies using the streptavidin/biotin-coated ELISA (top) and the directly coated ELISA (bottom). The following rhEPO concentrations were used: 0, 0.5, 5, 10, 50, 75, 100, 300 ng/ml. The inhibition was fitted by Eq. (1) (see Materials and methods).  $IC_{50}$  values were calculated based on the concentration of rhEPO at 50% inhibition.  $IC_{50}$  in streptavidin/biotin-coated ELISA: Calibrator—1583 pM; serum no. 223–273 pM; serum no. 351—289 pM;  $IC_{50}$  in directly coated ELISA: Calibrator—3877 pM; serum 223–300 pM; serum 351–49 pM.

Table 2

Comparison of anti-EPO antibody levels (ng/ml) in human sera when assayed by a streptavidin/biotin-coated ELISA using different incubation conditions

Serum no.	Streptavidin/biotin-coated ELISA (RT, 2 h)	Streptavidin/biotin-coated ELISA (37 °C, overnight)
1	2.3	2.5
2	5.3	10.7
3	5.7	9.1
4	8.6	19.1
5	9.4	22.6
6	10.5	8.7
7	19.1	17.8
8	33.2	58.4
9	55.6	66.2

### 3.4. Improvement of sensitivity of streptavidin/biotin-coated ELISA

The ELISA described by Hoesel et al. (2004) exhibited an LLD of 1 ng/ml and a lower limit of quantification (LLQ) of 2 ng/ml using immunopurified rabbit polyclonal anti-EPO IgG as reference material in the standard ELISA protocol with 10% serum in the incubation solution. In order to improve sensitivity, several approaches were investigated. Firstly, serum concentration in the assays was increased up to 50% of the incubation solution. This resulted in a linear response without influencing the background and achieved a five-fold lower LLQ for human sera compared to the standard conditions. Secondly, changes in the incubation conditions from RT and 2 h to overnight at 37 °C resulted in an average 50% increase in anti-EPO antibody detection. Notably, however, some sera increased whereas others remained the same (see Table 2). Thirdly, inclusion of two forms of rhEPO each digoxigenylated via different linkages (to the sialic acids of the carbohydrate groups of rhEPO or to amino groups) in the detection conjugate did not result in any improvement of the sensitivity. Thus, an increase in the sensitivity of the streptavidin/biotin-coated ELISA could be obtained for some sera with low activity by increasing serum concentration up to 50% and extending sample incubations overnight. This would allow an even higher sensitivity for the analysis of serum from patients with very low anti-EPO antibody levels.

## 4. Discussion

Our findings show that the method of rhEPO immobilization to microtiter plates is a critical determinant for the sensitivity of ELISAs used for measuring

anti-EPO antibodies. Capturing antibodies via streptavidin/biotin-immobilized rhEPO results in a sensitivity of up to two orders of magnitude higher than direct coating of rhEPO. It is, therefore, inappropriate to draw general conclusions that ELISAs are less sensitive for the analysis of anti-EPO antibodies compared to other assays such as RIPA, a view entirely based on a single report (Casadevall et al., 2004; Swanson et al., 2004; Swanson, 2003; Thorpe and Swanson, 2005; Locatelli et al., 2004). A double antigen bridging ELISA employing streptavidin/biotin coating would have very likely succeeded in detecting the two positive sera which Swanson et al. (2004) failed to detect. The sensitivity of the assay described by us (Hoesel et al., 2004) could also be further improved by increasing the concentration of serum in the assay and alterations of the incubation conditions as demonstrated above, resulting in more options to improve sensitivity when analysing low titer sera.

The marked differences between results for streptavidin/biotin-coated and directly coated ELISAs most likely result from the structure of the immobilized rhEPO (antigen). Passive coating appears to alter the structure of rhEPO and the accessibility of binding sites for specific antibodies. This is consistent with the findings of Butler et al. (1992) and Butler (2000) who reported that the pH conditions (pH 9.6) widely used for passive adsorption of antigen in microtiter ELISAs, can lead to protein denaturation and destruction of up to 90% of the functional activity of liquid-phase reactant. rhEPO would be particularly sensitive to this, being a comparatively hydrophilic protein with 40% carbohydrate content. Our data show that such a loss of functional activity can be significantly reduced by immobilization of rhEPO through streptavidin bridging (Butler et al., 1992) and indeed, the streptavidin/biotin-coated ELISA demonstrated a clearly enhanced antibody-binding capacity. It is worthy of note that  $IC_{50}$  values of rhEPO-positive sera showed comparable values with both ELISA coating methods, thereby arguing against differences in antibody binding affinities accounting for the low sensitivities observed with the directly coated ELISA.

Remarkably, quantitation of purified rabbit polyclonal antibodies is less dependent on the method used to immobilize rhEPO. This suggests a difference in binding sites for rabbit compared with human polyclonal antibodies as has been observed for ELISAs detecting antibodies specific for interferon- $\beta$  in serum from multiple sclerosis patients (Brickelmaier et al., 1999). Brickelmaier et al. reported that ELISAs in which IFN- $\beta$  was captured via a specific monoclonal antibody

or in which biotinylated IFN- $\beta$  was captured via streptavidin, detected serum antibodies that recognize IFN- $\beta$  in its native state. In contrast, ELISAs using directly coated IFN- $\beta$  detected antibodies that recognize alternate forms of IFN- $\beta$  possessing a folded structure distinct from the native structure. Moreover, peptide-binding assays demonstrated the presence of antibodies directed towards linear epitopes. These appeared to be present at the expense of antibodies recognising native IFN- $\beta$ , suggesting that certain epitopes of native IFN- $\beta$  had not been represented in these assays. As polyclonal rabbit antibodies are generated from an immunization procedure involving a harsh treatment with Freund's complete adjuvant, it is possible that there is some denaturation of the protein antigen, creating rhEPO epitopes similar to those resulting from direct coating in ELISAs. This would then generate rabbit anti-EPO polyclonal antibodies that would be detected in such an ELISA. In contrast, anti-EPO antibodies in human sera are more uniformly produced against the conformational epitopes of the native protein antigen and hence are less efficiently bound in directly coated ELISAs. Support for this hypothesis is found in a report by Casadevall et al. (2002), in which 13 out of 14 anti-EPO positive sera from patients with pure red cell aplasia patients reacted only with native, but not with denatured rhEPO.

A further influence on detection of anti-EPO antibodies may be variable accessibility to the antigen. All human serum assays with directly coated rhEPO showed a considerable increase in sensitivity upon overnight incubation (Table 1), but such an effect was less frequent and less marked among serum samples tested by streptavidin/biotin-coated ELISAs (Table 2). This suggests that antibody–antigen equilibrium is reached earlier with streptavidin/biotin-linkage compared with directly coated rhEPO, possibly due to less steric hindrance around the surface which results in an improved accessibility for building antibody bridges. The diversity of antibody affinities in human sera may further exaggerate the observed effect as lower affinity antibodies depend on avidity to support bridging of antibodies and therefore take longer to reach binding equilibrium.

In conclusion, the streptavidin/biotin-coated ELISA is a favorable system for the detection of anti-EPO antibodies in human sera whereas the directly coated ELISA is less well suited for that purpose. The high sensitivity of the streptavidin/biotin-coated ELISA, coupled with its practicality (in comparison to RIPA for example), makes it an attractive system

of choice particularly when screening large numbers of samples.

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