Assays for detecting and diagnosing antibody-mediated pure red cell aplasia (PRCA): an assessment of available procedures

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Abstract
Antibody (Ab)-mediated pure red cell aplasia (PRCA) develops when patients mount a neutralizing Ab response to recombinant erythropoiesis-stimulating agents (ESAs) such as epoetin-α (EPO). These neutralizing Abs can also cross-neutralize endogenous EPO, leading to a state of absolute EPO resistance and transfusion dependence. The diagnosis of Ab-mediated PRCA in part relies on the sensitive and specific detection of serum anti-EPO Abs and a confirmatory examination of patients' bone marrow for lack of erythroid precursors. To date, a variety of assays have been used to detect anti-EPO Abs, including radioimmunoprecipitation (RIP) assay, enzyme-linked immunosorbent assay (ELISA), surface plasmon resonance (SPR) and bioassays that measure neutralizing Abs. Each of these assays can yield informative results and possess characteristic benefits and limitations, so it is unclear whether or not a 'superior' assay is required or possible. To date, a universal standardized assay has not yet been established that would facilitate the comparison of Ab data derived from different laboratories and retrospective analysis of stored sera. This review evaluates the results of studies measuring anti-EPO Abs with different assays and compares their relative advantages and disadvantages in terms of specificity, sensitivity, ease of use and ability to measure Ab-binding affinities and subclasses. These comparisons provide a basis for determining the optimal assay(s) for screening and/or analysis of patients’ serum for anti-EPO Abs during treatment or after onset of Ab-mediated PRCA.

Keywords: assay; erythropoietin; neutralizing antibodies; pure red cell aplasia

Introduction
Patients with chronic kidney disease (CKD) frequently develop anaemia because of loss of endogenous erythropoietin (EPO) production by kidney interstitial cells. Administration of recombinant erythropoiesis-stimulating agents (ESAs) compensates for the loss of endogenous EPO production in these patients. Although treatment with ESAs is well tolerated in the majority of patients, a small number generate antibody (Ab) responses to ESAs that can effectively neutralize both circulating endogenous EPO and the recombinant protein(s). In some cases, production of anti-EPO Abs can lead to the development of pure red cell aplasia (PRCA) and severe, transfusion-dependent anaemia. Ab-mediated PRCA is confirmed diagnostically by the total or near absence of red blood cell (RBC) precursors in bone marrow that is otherwise normocytic and normochromic [1,2]. In addition, Ab-mediated PRCA is typically accompanied by extremely low levels of serum EPO, which contrasts with the elevated levels of serum EPO often observed in patients with non-Ab-mediated forms of PRCA. A more complete description of Ab-mediated PRCA is provided in the article by Casadevall, in this supplement.

Here we review the various assays that have been used to detect Abs that inhibit EPO-stimulated erythroid cell development, or Abs that bind to epitopes on the EPO molecule. Each assay has its own particular strengths and weaknesses for analysing these Abs quantitatively or qualitatively. A discussion of the optimum characteristics for a standard Ab assay is contained in a separate section of this review. A more complete understanding of the types and characteristics of the various available Ab assays may assist in the selection or design of such assays. It is not yet clear whether or not selection of a universal assay for detecting anti-EPO Abs is necessary or possible. In the absence of a designated standard assay, information provided in this review may assist physicians and scientists in selecting methods for detecting Abs...
that are clinically relevant to onset and progression of Ab-mediated PRCA.

Immuneogenicity of different ESAs

Between 1998 and December 2003, 201 confirmed cases of Ab-mediated PRCA were reported in patients receiving one or more ESA. The majority of patients had received subcutaneous (s.c.) injections of a certain formulation of epoetin-α distributed outside of the USA (Eprex®, Ortho Biotech LLC, Manati, Puerto Rico) given either alone (n = 180) or in combination with another ESA (n = 21) [3]. Additional cases of Ab-mediated PRCA were reported in patients receiving epoetin-β [4] s.c. (n = 8) [5] or another formulation of epoetin-α manufactured by Amgen and marketed in the USA (Epogen®/Procrit®, Amgen Inc., Thousand Oaks, CA/Johnson & Johnson, New Brunswick, NJ, USA) (n = 5) [6]. No cases of PRCA have been reported in patients treated exclusively with darbepoetin-α (Aranesp®, Amgen Inc.) [7,8]. It is not known definitively which factor(s) induce the production of anti-EPO Abs, but, regardless of the identity of the immunogenic culprit, patients with Ab-mediated PRCA develop resistance to endogenous EPO and all recombinant ESAs. Therefore, upon confirming the presence of epoetin-binding Abs and PRCA, it has been recommended that treatment with any ESA should be immediately discontinued [9].

Desirable characteristics of diagnostic antibody assays

A useful diagnostic Ab assay should possess the features of versatility, sensitivity, specificity and reproducibility. Versatility in an Ab assay includes the ability to quantify EPO-specific Abs (most important feature), the ability to determine Ab isotypes qualitatively and the ability to measure Ab affinities for EPO or its epitopes and related molecules. A versatile assay can therefore provide a number of useful quantitative and qualitative measures of specific Abs. Another desirable feature of a useful Ab assay is sensitivity, which is the ability to measure very low concentrations of specific Abs while exhibiting low or very low background signal. Assay specificity refers to the ability to measure Abs that bind specifically to the EPO protein or epitope being studied.

Assays for EPO Abs should not detect non-EPO Abs, but such an assay may detect Abs that cross-react with the various ESAs because of their biochemical and immunochemical similarity to each other and to endogenous EPO.

Assays for anti-EPO antibodies

Since the 1960s, a variety of methods have been used to study Abs associated with PRCA. These methods include inhibition of haem synthesis in normal bone marrow [10], immunocytofluorescence [10], cytotoxic release of $^{59}$Fe from labelled erythroblasts [11], radioimmunoprecipitation (RIP) [2,12–15], enzyme-linked immunosorbent assay (ELISA) [15–17], surface plasmon resonance (BIACore) [15], and bioassays that measure proliferation of EPO-dependent primary erythroid cells or cell lines [13,15].

Current assays for epoetin Abs include RIP, ELISA, BIACore and bioassays. Each type of assay has particular advantages and disadvantages in terms of sensitivity, specificity, ease of use, and so on. Various ELISA formats have been used for the analysis of proteins, small molecules and Abs, including direct, indirect, competitive, sandwich and bridging ELISAs. The ELISAs are quite reproducible and easy to perform and, because they rely on Abs for capture and/or detection of proteins, they can also deliver high specificity. In contrast, bioassays are the only laboratory methods capable of measuring EPO-neutralizing Abs that functionally inhibit cellular proliferation and erythroid colony formation. A summary of the advantages and disadvantages of each of these Ab assay platforms is shown in Table 1.

ELISA

Urra and colleagues [16] developed an indirect ELISA (Figure 1) for analysing serum from an EPO-resistant haemodialysis patient, 10 epoetin-treated haemodialysis patients without EPO resistance and 30 normal controls. Serial dilutions of patients’ plasma were incubated in wells of microtitre plates that were coated previously with either epoetin-α or epoetin-β, followed by the addition of a secondary, labelled Ab to detect anti-EPO Abs bound to the immobilized epoetin. Their results showed detectable IgG EPO Abs but no IgM Abs in serum from the single EPO-resistant patient. Antibodies bound to both epoetin-α- and epoetin-β-coated wells and also neutralized EPO in a bioassay. No Abs were detected in serum from normal controls or the 10 haemodialysis patients.

Swanson and colleagues [15] compared four different Ab assays for detecting anti-EPO Abs in sera from 13 patients with Ab-mediated PRCA; results on eight of the 13 sera were reported. These investigators utilized a bridging ELISA in which single antigen-binding sites on serum Abs attach to plate-immobilized epoetin, leaving the second antigen-binding sites available to bind subsequently to free, labelled EPO added later in the procedure. With this assay, two of the eight tested samples yielded false-negative results, based on comparative results obtained by RIP. The sensitivity of this assay for anti-EPO Abs was reported as 900 ng/ml of serum.

Together, these results suggest that some ELISAs may not possess the levels of specificity and sensitivity required for a standard method to detect potentially low levels of EPO-specific Abs, or Abs associated with PRCA.
The first RIP assay for EPO made widely available was described by Egrie and colleagues in 1987 [12]. These investigators were the first to utilize recombinant human EPO and Abs specific to the recombinant EPO as standard reagents for the RIP assay; these standards replaced urine-derived EPO (U-EPO) and anti-U-EPO Abs that had been used in previous assays. This RIP assay or variations of this assay were used subsequently by several investigators to detect anti-EPO Abs in patients presenting with Ab-mediated PRCA [13,18,19].

In the RIP assay, serum Abs are allowed to bind to 125I-labelled epoetin in solution, and the resulting complexes are captured by a solid-phase anti-globulin reagent (e.g. protein A-Sepharose beads) and pelleted by centrifugation for analysis (Figure 2). The amount of radioactivity in the pelleted samples corresponds positively with the relative concentration of EPO-specific Abs in the serum samples. This method offers...
a number of advantages, including ease of use, specificity for high-affinity IgGAbs, and excellent sensitivity (Abs bind in as little as 200 mIU of EPO/ml of serum) [2,14]. Casadevall and colleagues also used the RIP assay to determine that anti-EPO Abs in patients with PRCA bound to the protein core of the EPO molecule, and not to the carbohydrate side chains. In addition, the RIP assay proved useful in Scatchard analysis of the binding affinity of IgG Abs for EPO [13].

The disadvantages of the RIP assay include required use of a radionuclide (125I) and poor or unlikely detection of low-affinity Abs, thus making this method unsuitable for detecting Abs produced during the early stages of PRCA. However, a modified RIP used by Swanson and colleagues allows for detection of IgM Abs specific for EPO [20]. This assay is also highly sensitive, with a detection limit of 10 ng/ml of anti-EPO Abs.

**BIAcore**

Surface plasmon resonance using the BIAcore technology is a versatile technology for detecting anti-EPO Abs. This method relies on the change in the angle of incident light reflected off the gold surface of a biosensor chip as a function of the amount of protein mass accumulating on the sensor chip surface (Figure 3A). Any specific Abs in serum will bind quickly to a recognized antigen (eg. EPO) already bound to the sensor chip surface, thus increasing the protein mass and altering the angle of reflection of light reaching the photometric detectors. The binding of Abs on the biosensor surface is expressed graphically in sensorgrams that depict accumulation of protein over time, and the loss of protein during wash out periods that serve to remove non-specific Abs or low-affinity Abs at certain rates (Figure 3B). Consequently, the BIAcore assay provides real-time cumulative Ab binding data, rather than a single end-point, thus allowing for direct determination of the relative binding affinities of patients' Abs. In addition, the BIAcore also enables quantitative measurements of Ab dissociation rates and classification of Ab isotypes, based on the use of isotype-specific, secondary Abs. Other advantages of this method include the ability to reuse sensor chips for serial Ab analysis and no use of radionuclides or antiglobulin reagents or need for their preparation.

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**Fig. 3.** (A) Diagramatic representation of the principle governing the manner in which the BIAcore assay detects specific antibodies. (B) The sensorgrams of typical low- and high-affinity antibodies. Diagram provided courtesy of Amgen Inc. Data on file.
The major drawback to use of BIACore technology is that the equipment is costly.

Swanson and colleagues used BIACore analysis to measure EPO Abs in eight of 13 patients with Ab-mediated PRCA [20], as well as a study of baseline Abs in 1501 treatment-naive and epoetin-experienced patients enrolled in a phase III/IIIb clinical trial with darbepoetin-α [15]. In the former study, complete concordance was obtained between the BIACore results and titres calculated with RIP and a bioassay. In addition, the relative Ab binding affinities (expressed as percentage reduction in accumulated Ab protein) and isotypes (predominantly IgG1 and IgG4) were determined for each of the eight PRCA sera [20]. However, two of the eight sera tested negative for EPO Abs using an ELISA platform, leading to the conclusion that the ELISA yielded false-negative results with these two PRCA sera.

In the latter study of 1501 baseline sera, Abs binding to epoetin-α (n = 22), darbepoetin-α (n = 21) or both (n = 16) were detected in 59 serum samples [15]. Antibody subclasses were also determined in 27 of the 59 serum samples: 11 of the 22 anti-epoetin sera (seven IgG, three IgM and one IgG1 IgM), and 16 of the 21 anti-darbepoetin sera (seven IgG, eight IgM and one IgG1 IgM). In this study, the BIACore assay also demonstrated excellent sensitivity levels (80–100 ng/ml of serum Abs) and could detect low-affinity Abs. However, the 59 BIACore-positive sera produced negative results in both a bioassay (having relatively low sensitivity) and RIP (high sensitivity). Therefore, the BIACore assay was measuring low-affinity Abs in baseline sera. None of the patients developed Ab-mediated PRCA or any significant treatment-related pathology, and the clinical relevance of baseline Abs to the ultimate development of PRCA has yet to be demonstrated.

Bioassays

While ELISA, RIP or BIACore may be used initially to test patients’ serum for the presence of Abs that bind to EPO, none of these methods can demonstrate neutralizing activity—an important aspect for diagnosis of Ab-mediated PRCA. Demonstration of neutralizing activity requires a bioassay, in which bone marrow-derived erythroid cells or certain cell lines are stimulated to proliferate or form colonies in the presence of exogenous EPO. This EPO-dependent cellular proliferation is blocked by neutralizing Abs present in serum from patients with epoetin-induced PRCA (Figure 4).

Cell lines offer some advantages over primary bone marrow cells for EPO bioassay. These include greater reproducibility between assay runs and consistent cell growth characteristics and EPO responsiveness. Bone marrow samples may be available in limited quantities, and the assay involves cultivating the marrow samples for up to 14 days, followed by fixing and staining of samples and quantification of erythroid colony formation. Three cell lines that have been used to demonstrate epoetin-neutralizing activity are TF-1 and UT-7, which are both cell lines derived from patients with erythroleukaemia, and 32Dc13.sccl2, a murine haematopoietic cell line transfected with the receptor for human epoetin. In the presence of EPO-neutralizing Abs, UT-7 and TF-1 cells will not proliferate in response to exogenous EPO [13]. Increasing concentrations of exogenous epoetin reverse the neutralizing effect of serum Abs in the bioassay. The specificity of these bioassays is demonstrated by the continued proliferation of cells after addition of granulocyte-macrophage colony-stimulating factor (GM-CSF) that is not inhibited by anti-EPO Abs. In these bioassays, normal serum does not inhibit EPO-stimulated cellular proliferation. Similarly, 32Dc13.sccl2 cells proliferate in response to either epoetin or murine interleukin-3 (mIL-3), and neutralizing Abs that inhibit cellular proliferation induced by epoetin do not inhibit proliferation induced by mIL-3 [20].

Although bioassays are clearly the only assays that can measure functional EPO-neutralizing Abs, the disadvantages of the assay include long incubation times (days) and moderate sensitivity in some cases. In addition, as shown in Figure 5, different EPO-sensitive cell lines have different inherent sensitivities to the neutralizing effects of anti-EPO Abs in the bioassay. This can result in varying results with a single serum sample that may be tested in a bioassay using different cell lines. The bone marrow proliferation or erythroid colony formation assay may also be less robust since non-specific serum factors could influence proliferation. Nevertheless, bioassays are the cornerstone for defining anti-EPO neutralizing activity in serum.

Need for a standard assay

The increased incidence of Ab-mediated PRCA underscores the need for reliable tests to detect and characterize Abs in the serum of CKD patients who are at risk for this condition. Although a variety of assays have been used to this end, no single assay suffices for both detecting and fully characterizing the presence of Abs in serum and determining their neutralizing
Sensitivity of EPO responsive cell-lines to EPO Abs

Fig. 5. Varying sensitivities of different bioassay cell lines to the neutralizing effects of anti-EPO serum antibodies.

Table 2. Characteristics of the four assays used to measure anti-EPO antibodies

<table>
<thead>
<tr>
<th>Assay</th>
<th>Reference</th>
<th>Lower limit of sensitivity</th>
<th>Measures Ab isotypes</th>
<th>Measures Ab affinities</th>
</tr>
</thead>
<tbody>
<tr>
<td>RIP</td>
<td>[21]</td>
<td>0.21U EPO bound/ml serum</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>ELISA</td>
<td>[20]</td>
<td>780 ng (78 IU) EPO bound/ml serum</td>
<td>Yes, in some cases</td>
<td>Not usually</td>
</tr>
<tr>
<td>BIAcore</td>
<td>[20]</td>
<td>80–100 ng/ml serum Abs</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Bioassay</td>
<td>[21]</td>
<td>1 IU EPO bound/ml serum</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

Capabilities. Each Ab assay has its own particular level of sensitivity and versatility for detecting Ab isotypes or binding affinities, for example (Table 2). At least two assays must be used for detecting and characterizing EPO Abs, an assay to determine the existence of serum Abs that bind to EPO and a bioassay to demonstrate the Abs' ability to inhibit the biological activity of epoetin in living cells. Various laboratories have used different assays for detecting anti-EPO Abs, based on the advantages and drawbacks of each. However, variability in the sensitivity and specificity of the different assays makes it difficult to compare directly the test results obtained in different laboratories. Identification of an assay which is clearly superior to others in all aspects is not easy and may be impossible. However, the availability of standardized reagents and reference preparations is invaluable for assay validation and comparison.

Conclusions

The ability of different assays to measure clinically relevant EPO Abs is still a matter of discussion among researchers, and a consensus has not been reached on which assay is most meaningful and appropriate for screening of certain patients’ serum, and which is most meaningful in terms of predicting onset of Ab-mediated PRCA and its clinical progression. It may, in fact, be difficult or impossible to establish a universal assay for anti-EPO Abs because of lack of acceptance among different users, lack of availability of critical reagents or equipment, or other reasons. Nevertheless, validation and qualification of assays for measuring and characterizing anti-EPO Abs is essential to obtaining meaningful assay results. The availability of well characterized reagents and reference preparations is also important to establishing a standardized assay. The distribution of reference panels of control sera would promote the utilization of one or more standardized assay and would be very helpful for assay validation and performance evaluation.

Appropriate Ab binding assays that meet the difficult criteria of versatility, sensitivity and specificity will prove useful in detecting and characterizing anti-EPO Abs in patients with confirmed Ab-mediated PRCA.

Note added in proof: As of March 2005, 2 suspected cases of PRCA in patients treated with darbepoetin-alpha alone are under investigation.

Conflict of interest statement. None declared.
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