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Brief Communication

Immunogenicity of Recombinant Human Erythropoietin: Clinical Cases, Causes and Assays

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Human erythropoietin (EPO) is a glycoprotein that enhances red blood cell production by stimulating proliferation and differentiation of erythroid progenitor cells in the bone marrow. Patients with chronic kidney disease (CKD) suffer from anemia caused by reduced production of EPO in the kidney. Recombinant human EPO protein has been used successfully for the treatment of anemia associated with CKD. Recently, attention has been paid to the development of side effect of EPO, pure red cell aplasia (PRCA), in some patients with CKD. PRCA is a rare disorder of erythropoiesis that leads to a severe anemia due to an almost complete cessation of red blood cell production. EPO-related PRCA is caused by the production of EPO-neutralizing antibodies (Abs) that eliminate the biological activity of EPO as well as endogenous EPO in patients undergoing therapy. Since 1988, almost 200 cases worldwide have been reported with Ab-positive PRCA after receiving EPO therapeutics. The underlying mechanisms of the breaking of immune tolerance to self-EPO have been investigated. Modification of formulation, organic compounds of container closures, and route of administration has been suggested for the possible mechanism of increased immunogenicity of EPO. A number of assays have been used to detect Abs specific to EPO. These assays are generally grouped into two major categories: binding Ab assays and neutralizing Ab assays (bioassays). There are several types of binding Ab assays, including radioimmunoprecipitation assay, enzyme-linked immunosorbent assay, and the BIAcore biosensor assay. In vitro cell-based bioassays have been utilized for the detection of neutralizing Abs. Finally, the recent experience with anti-EPO Abs may have considerable implications for the future development and approval of EPO preparations. Also, considering that millions of patients are being treated with EPO, clinicians need to be aware of signs and consequences of this rare but severe clinical case.

Key Words: Erythropoietin, EPO, PRCA, Immunogenicity

Clinical cases of EPO immunogenicity

EPO is a polypeptide consisting of 165 amino acids and is a heavily glycosylated protein with a molecular mass of 30,400 Daltons. Glycosylation process is essential to the biological activity of the molecule. About ninety percent of EPO is produced in the peritubular cells of the kidney and enhances red blood cell production by stimulating proliferation and differentiation of erythroid progenitor cells in the bone marrow. Since 1988, CKD patients with anemia caused by reduced production of EPO in the kidney have been treated with recombinant EPO. Now, recombinant EPO is the largest therapeutic protein class in the world ($10,212 million, 2006) (Datamonitor, 2002).

From 1988 to 1997, neutralizing anti-EPO Abs have been reported in three CKD patients. However, additional 212 Ab positive cases were reported by 2005 (Johnson & Johnson Pharmaceutical Research & Development, 2008). 189 cases were occurred by EPREX® exposure only and 24 cases were occurred by exposure to another erythropoietin and EPREX® (Table 1).

In 2002, 13 cases of EPO-associated pure red-cell aplasia (PRCA) were reported (Casadevall et al., 2002). In this

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Table 1. Summary of PRCA Case Reports

Status of Reports in Chronic Renal Failure Patients		Year Unknown	Prior to 1998	1998	1999	2000	2001	2002	2003	2004	2005	Total
Antibody-Positive PRCA	EPREX®* Exposure only	3	3	5	8	24	62	68	12	4	2	189
	Exposure to another erythropoietin and EPREX®	1	0	1	0	4	5	5	6	2	0	24
Total Antibody-Positive PRCA		4	3	6	8	28	67	73	18	6	2	215

*Recombinant human erythropoietin alpha (Johnson & Johnson, Manti, Puerto Rico)

Table 2. Current standard diagnostic criteria for PRCA (Casadevall et al., 2004)

- Fall in red cell count of about 1%/d
- Reticulocyte count below 1%
- No major changes in white cell count, platelet count, or differential leucocyte count
- Normal cellularity of bone marrow
- Less than 1% erythroblasts (occasionally up to 5% proerythroblasts or basophilic erythroblasts)
- Normal myeloid cells and megakaryocytes

report, PRCA was observed in renal anemia patients treated with subcutaneously administered EPO. Nine out of 13 PRCA patients were treated with erythropoietin alpha, one with erythropoietin beta, two with erythropoietin beta prior to erythropoietin alpha, and one with erythropoietin alpha prior to erythropoietin beta. Erythropoietin alpha has more sialic acid residues than erythropoietin beta. This rare condition of PRCA is caused by the production of EPO-neutralizing Abs that eliminate the biological activity of the recombinant EPO protein as well as of endogenous EPO. PRCA is a severe anemia with almost complete absence of red blood cell precursors in the bone marrow (Krantz, 1974). Possible major causes of PRCA except for EPO-treatment are lymphoproliferative disorders, infections, systemic autoimmune disease, drugs, thymoma, and idiopathic (Eckardt and Casadevall, 2003).

When CKD patients are refractory to EPO-treatment for anemia, one should check the signs of iron deficiency, vitamin B12 deficiency, folate deficiency, hyperparathyroidism, bleeding, inflammation, hemolysis or aluminum overload. If these clinical signs are not found, one should doubt EPO-associated PRCA and confirm by bone marrow aspirate examination and serum assays for antibodies. According to the current standard diagnostic criteria (Table 2), EPO-associated PRCA is characterized by severe anemia and an almost complete absence of red blood cell precursors and patients become heavily transfusion-dependent (Casadevall et al., 2004).

Once PRCA was diagnosed, immunosuppressive treatment was frequently initiated. Immunosuppressive regimens are prednisone, intravenous immune globulin (often with prednisone), cyclophosphamide (often with prednisone), or cyclosporine (Table 3).

Little cases of PRCA have been reported in Korea. Recently, two cases of PRCA due to anti-erythropoietin Abs were reported (Yang et al., 2005). The first patient had anti-EPO Abs detected by ELISA and radioimmunoprecipitation and these were found to inhibit erythroid colony formation by *in vitro* bioassay. The second patient also had anti-EPO Abs but *in vitro* bioassay could not be performed because of sudden death of patient. These cases demonstrate that EPO-associated PRCA becomes an important issue in Asians as well as in Caucasians.

Factors influencing immunogenicity of EPO

Many factors are reported to influence the immunogenicity of therapeutic proteins (Table 4) (Schellekens, 2002). Generally, therapeutic proteins derived from non-human origin are immunogenic. Also non-glycosylated

Table 3. Immunosuppressive regimens used for treatment of epoetin-associated pure red cell aplasia (Bennett et al., 2005)

Immunosuppressive treatments	Dose range	Route	Observed recovery, %*
Cyclophosphamide + prednisone	50~100 mg/day + 1 mg/kg/day	Oral	87
Cyclosporine	100 mg twice/day or 5~8 mg/kg/day	Oral	67
Prednisone	1 mg/kg/day	Oral	56
Intravenous immunoglobulin*	2 g/kg over 2 to 5 days	Intravenous	11

*Recovery rates are based on long-term follow-up reported by the European PRCA Study Group for 47 patients with complete follow-up data (Verhelst et al., 2004)

Table 4. Factors affecting immunogenicity of therapeutic proteins (Gribben et al., 1990)

Factor	Potential Effects
Genetic background	Patient's immune defect may indicate a lack of natural immune tolerance
Type of disease	Antibody production may be increased by infectious diseases or reduced by immunosuppression
Type of protein	Nonhuman molecules are usually more immunogenic
Conjugates	Could create new antigenic determinants
Fragments	Could expose new antigenic epitopes
Route of administration	SC most immunogenic; IM less so; IV least immunogenic
Dose frequency	Immunogenicity increases with more frequent dosing
Duration of treatment	Short-term generally less immunogenic than long-term
Manufacturing process	May introduce impurities or alter 3-dimensional structure (eg, via oxidation or aggregation)
Handling and storage	May alter 3-dimensional structure (eg, via oxidation or aggregation)

form of protein such as GM-CSF expressed in prokaryotic cell line could increase the immunogenicity by epitope exposure (Gribben et al., 1990). In case of human growth factor and insulin, impurities, contaminants, and aggregates were regarded as main cause of antibody production (Palleroni et al., 1997). Properties of the product formulation are included as product-related factors as well.

Patient's factors are also important (Schellekens, 2003). Genetic background, such as patient's immune defect, may indicate a lack of natural immune tolerance. MHC polymorphism can play a crucial role in regulating the presentation of antigenic peptide for T cell recognition and in immunogenicity. As for the Factor VIII, genetic mutation of Factor VIII is more important determinant than MHC polymorphism. The incidence of antibodies is higher in viral infected patients than in immunocompromised cancer patients suggesting the importance of disease state in immunogenicity.

Route of administration is another factor and subcutaneous administration showed a higher incidence of immunogenicity than intravenous route. Except for these factors, it appears that there are a number of unknown factors influencing immunogenicity of therapeutic proteins.

The incidence of PRCA began to increase in 1998 and peaked in 2002 (Boven et al., 2005a). Apparently, this rise in incidence appears to be associated with subcutaneous application of EPO and slight change of formulation. A relative minor change for Eprex® was replacing human serum albumin as a stabilizer with the detergent polysorbate 80 in order to comply with new regulations from the European regulatory authorities (Eckardt and Casadevall, 2003).

Eprex's manufacturer, Johnson & Johnson, recently suggested another immunogenicity factor. They have claimed that the increase in immunogenicity of epoetin alpha was due to an adjuvant effect of compounds leached from the rubber syringe stoppers by polysorbate 80 (Boven et al., 2005a, Boven et al., 2005b, Schellekens and Jiskoot, 2006). In contrast, this result evoked a criticism that the increased incidence was not caused by an effect of the leachates which break B-cell tolerance via an adjuvant effect because even strong adjuvants have failed to break tolerance (Ottesen et al., 1994, Schellekens and Jiskoot, 2006, Stewart et al., 1989).

Alternatively, aggregates were regarded as an explanation

for the EPO-associated PRCA cases (Schellekens and Jiskoot, 2006). EPO shows an increase in the levels of aggregates during storage. The increased incidence of PRCA with Eprex compared with epoetin alpha produced in the US suggests intrinsic differences. This suggests an increased tendency for aggregation, which may be aggravated by an interaction with the polysorbate 80 (Schellekens and Jiskoot, 2006). Besides the prohibition of the subcutaneous route of administration, the improved maintenance of the cold chain becomes the main reason for the reduction of cases.

Assays for anti-EPO antibodies

The clinical significance of antibodies against EPO has been emphasized by a recent increase of PRCA cases. Several assays are currently used to test the presence of antibodies, including enzyme-linked immunosorbent assay (ELISA), radioimmunoprecipitation (RIP), surface plasmon resonance (SPR) and bioassays measuring neutralizing antibodies. To date, however, a standard assay has not yet been established that would enable different laboratories' data to be compared.

To perform the RIP assay, clinical serum samples containing antibodies are allowed to bind to ^{125}I-radiolabeled EPO in solution. And protein A or G-sepharose beads are added to the EPO-antibody complex solution. Centrifugation captures the radioactive complex to a pellet. The amount of radioactivity in this sample is measured by a gamma counter. This method has some advantages, including high sensitivity, low cost, ease of use. The disadvantages of RIP are low throughput assay, requiring the use of radioactive material, specificity of Ab isotype, and poor detection of low-affinity antibodies (Thorpe and Swanson, 2005a).

Anti-EPO antibodies are detectable by indirect or bridging ELISA. Variations of these assays have been developed to improve sensitivity and specificity (Thorpe and Swanson, 2005a). Indirect ELISA detects serum Abs that bind to EPO proteins coated on plastic plates by using enzyme-conjugated secondary Abs and its substrates. To reduce nonspecific background, optimization is needed in indirect ELISA. In bridging ELISA, a bridge is formed between EPO immobilized on plastic wells and enzyme-labeled EPO via anti-EPO antibodies. Bridging ELISA reduce background readings by using fewer amplification steps in the procedure and the requirement for two specific binding events for the target EPO, which increases specificity of the assay. This method is rapid, easy, inexpensive, high throughput, and highly specific. However, this method can give non-specific matrix effects and may fail to detect low-affinity antibodies. Recently, the new double antigen bridging ELISA was developed (Swanson et al., 2004). Anti-EPO Abs in CKD patients' sera bind via one antigen binding site to biotinylated-EPO immobilized to streptavidin-coated microtiter plates and via second site to digoxigenylated-EPO. The amount of bound anti-EPO Abs is determined by an anti-digoxigenin Ab conjugated to peroxidase (Fig. 1). This assay was very sensitive (lower limit of detection 1 ng/ml), specific (no backgrounds in normal serum samples) and suitable for screening large numbers of samples.

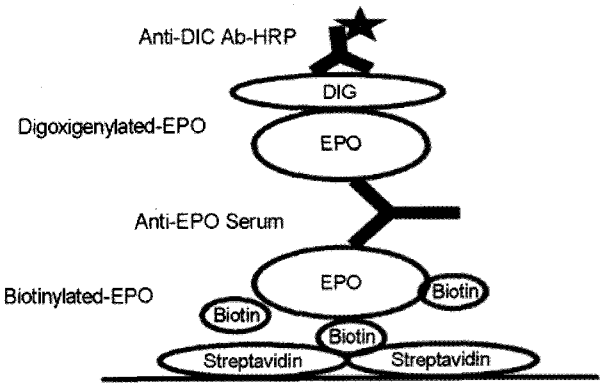

Fig. 1. Schematic diagram of anti-EPO double antigen bridging ELISA for analyzing of anti-EPO antibodies in human sera.

Surface plasmon resonance using the BIAcore is another technique for detecting anti-EPO antibodies (Thorpe and Swanson, 2005b). EPO protein is immobilized on the surface of sensorchip and serum samples are injected. When anti-EPO antibodies bind to EPO on the sensorchip, mass dependent signal is detected. By using this method, binding monitoring in real time, affinity measurement, and isotyping of the antibodies are possible. The major disadvantage of this assay is that it requires high cost and a skilled worker.

Although appearance of neutralizing anti-EPO Abs are important diagnostic aspect in Ab-mediated PRCA, RIP, ELISA, and SPR assays may be only used to test anti-EPO binding antibodies. To detect and characterize neutralizing

activity of anti-EPO antibodies, bioassay is required. Primary erythroid cells or certain cell lines are stimulated to form colonies or proliferate in the presence of recombinant EPO. Activity of recombinant EPO could be inhibited by patient serum sample if this contains neutralizing antibodies. Because primary cells grow slowly and finitely, immortalized or cancer cell lines have been utilized as alternative EPO responder cells. These cell lines include UT-7 cell (human erythroleukemia cell line) (Casadevall et al., 1996) and IL-3-dependent murine hematopoietic cell line 32D (Swanson et al., 2004). It has been reported that proliferation of UT-7 cells expressing a large number of EPO receptors was inhibited by PRCA patients' serum (Casadevall et al., 1996). IL-3-dependent murine hematopoietic cell line 32D was transfected by human EPO receptor gene to express EPO receptors on their cell surface. So this cell line could respond to human EPO as well as murine IL-3. Swanson *et al.* found anti-EPO Abs from the CKD patients' sera and checked their neutralizing activity with bioassay using murine 32D cell line (Swanson et al., 2004). Although neutralizing activity of serum samples can be determined by bioassay, it has some hurdles to be overcome. Each laboratory uses different EPO-responder cell lines, sensitivity of bioassay is not so high and scale-up and automation is rather difficult.

Currently, as various EPO Ab assays exist and are performed clinically in different worldwide labs, the comparison of data from each assay format is not sometimes easy and relevant. Therefore it is required that assays must be standardized. Standardization requires the development of appropriate biological standards for assay validation and calibration.

Finally, the recent experience with anti-EPO Abs may have considerable implications for the future development and approval of EPO preparations. Also, considering that millions of patients are being treated with EPO, clinicians need to be aware of signs and consequences of this rare but severe clinical case.

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