

Anti-Erythropoietin Antibodies and Pure Red Cell Aplasia

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More than twenty years ago, investigators who immunized rabbits against human erythropoietin (EPO) to generate polyclonal antibodies noted that the animals producing antibodies against this hormone became progressively anemic (1). Apparently human EPO was sufficiently different from rabbit EPO to be recognized as a foreign protein; however the antibodies were crossreactive with rabbit EPO and neutralized it. At that time the anemia observed in these animals provided indirect proof that EPO is an essential growth factor for erythropoiesis.

Patients have been treated with recombinant human EPO (rHuEPO or epoetin) since the late 1980s. Initially, the recombinant hormone was only given intravenously. Several years later the subcutaneous route of application started to become increasingly used, which facilitates self-administration and is believed to reduce dose requirements (2–4). In this clinical context, epoetin-induced antibodies remained a very rare complication for many years, and only few case reports have been published (5–7). Like in animals immunized against EPO the critical issue in patients developing anti-EPO antibodies is that these antibodies do not only abrogate the effect of the recombinant molecule, but also neutralize residual naturally occurring EPO. The resulting anemia is therefore much more severe than before the onset of therapy. Since 1998, the number of reported cases with severe, isolated inhibition of erythropoiesis [pure red cell aplasia (PRCA)] due to the development of anti-EPO antibodies has increased dramatically. This concerns almost exclusively patients with kidney disease receiving subcutaneous treatment with epoetin (8,9). To date, approximately 250 suspected or proven cases have been reported worldwide, the vast majority of those in association with the use of epoetin alfa outside the United States (10). Given that millions of patients are treated with epoetins, the prevalence of this complication remains very low, but the dynamics of the increase in its incidence have initially raised great concern. Meanwhile, causes and risk factors have become somewhat clearer. Speculations that antibodies against epoetin are a frequent and

unrecognized phenomenon have not been confirmed, and the incidence rates of epoetin-induced PRCA seem to have passed the peak. Nevertheless, clinicians should be aware of signs and consequences of this complication. Moreover, this issue raises important safety considerations for the development and approval of future epoetin molecules and biogenerics, in general.

Pure Red Cell Aplasia: A Rare Hematological Disease

PRCA is an isolated disorder of erythropoiesis that leads to a progressive, severe anemia of sudden onset (11). Due to an almost complete cessation of red blood cell (RBC) production the blood reticulocyte count is very low ($<10,000/\text{mm}^3$). The hemoglobin concentration declines at a weekly rate of about 1 g/dl, reflecting a decay that corresponds to normal RBC life span. To avoid very severe anemia and maintain an acceptable hemoglobin level, transfusion of about 1 unit of RBC per week is required. The bone marrow examination shows almost complete absence of erythroid precursors, but normal platelet and white cell precursors (Figure 1). A bone marrow aspirate is usually sufficient to make the diagnosis, but a biopsy may be necessary if the bone marrow smear is not optimal, particularly if cellularity is abnormal.

Factors known to be associated with PRCA include lymphoproliferative disorders and viral infections; in particular, those with parvovirus B₁₉, systemic autoimmune disease and drugs. However, about half of the cases have no identifiable cause (Table 1) (12). In many instances, the pathogenesis involves autoimmune mechanisms. Sera from affected patients were found to contain IgG antibodies that suppress *in vitro* growth of erythroid progenitors cells. In addition, inhibition of erythroid proliferation by T cells has also been shown to play a pathogenic role, even in patients with B cell lymphoproliferative disorders. Three cases have also been reported of patients never exposed to epoetin who developed PRCA due to the formation of autoantibodies against EPO (13–15). In the particular case of parvovirus B₁₉ infection, which is a known complication after kidney transplantation (16), the virus binds to a neutral glycosphingolipid, which is predominantly found on erythroid progenitors (blood group P antigen) (17). After infection it induces the formation of giant pronormoblasts and cell apoptosis.

Diagnosis and Clinical Signs of Epoetin-Induced PRCA

Diagnostic criteria for epoetin-induced PRCA are summarized in Table 2. Patients developing PRCA due to anti-EPO

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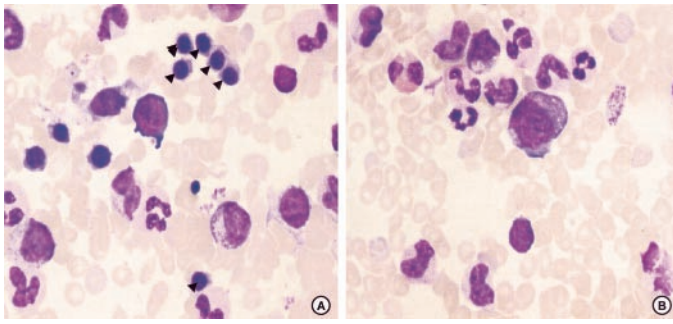


Figure 1. Bone marrow smear. (A) normal bone marrow, containing polychromatophilic erythroblasts (arrowheads). (B) bone marrow of a patient with PRCA

Table 1. Most frequent causes of PRCA

- Lymphoproliferative disorders
- Infections (e.g., parvovirus B19)
- Systemic autoimmune disease (e.g., systemic lupus)
- Drugs (e.g., chloramphenicol)
- Thymoma (in ~5% of cases)
- Idiopathic (in ~50% of cases)

Table 2. Diagnosis of epoetin-induced PRCA

- Major features
 - treatment with epoetin for at least several wk
 - weekly drop of hemoglobin of ~1 g/dl without transfusions or transfusion need of ~1 U per wk to keep hemoglobin levels in an acceptable range
 - reticulocytes < 10,000/mm³
 - no major drop of leukocyte and platelet counts
- Minor features
 - skin or systemic allergic reactions
- Confirmational investigations
 - bone marrow aspirate
 - normal cellularity and <5% erythroblasts
 - evidence for block of maturation
 - serum
 - presence of anti-erythropoietin antibodies

antibodies have typically been on epoetin therapy for 6 to 18 mo. The hemoglobin level then suddenly starts to decline at the rate indicated above, despite continued therapy with epoetin at the same or even increased doses (8) (Figure 2). The shortest time interval between start of epoetin therapy and loss of efficacy observed in a single case was 2 mo and the longest was 90 mo.

The reticulocyte count obtained by automatic flow cytometry is of great value in the differential diagnosis of the loss of efficacy of epoetin. Although great variation of this parameter is seen in patients with chronic kidney disease (CKD) receiving epoetin (Figure 3), values above 20,000/mm³ seem to exclude the diagnosis of PRCA. In a series of 45 patients with PRCA,

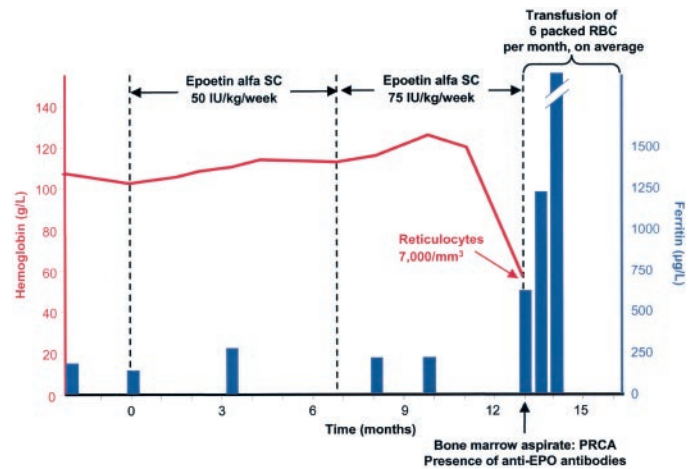


Figure 2. Schematic representation of a typical case of epoetin-induced PRCA. Red line: hemoglobin levels. Blue bars: ferritin levels.

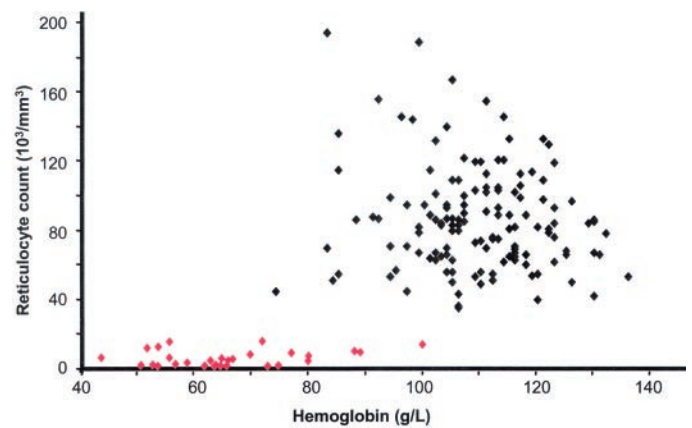


Figure 3. Reticulocyte counts in dialysis patients without PRCA (black dots) and in CKD patients with PRCA (red dots)

the median reticulocyte count was 3000/mm³ at the time of diagnosis (18). It has even been suggested that reticulocyte counts should be regularly monitored during epoetin therapy to allow early detection of impaired erythropoiesis (19). However, given the low incidence of epoetin-induced PRCA and absence of evidence that earlier detection will enhance outcome, this proposal has not been widely accepted.

Systematic analysis of cases reveals that when patients develop PRCA, they also experience a significant drop of platelet counts. Whether this is related to direct effects of EPO on megakaryopoiesis (20,21) or more complex interaction of platelet formation with erythroid precursor cells is unknown. In any case, the decline in platelet counts does not appear to be clinically significant, and in most cases thrombocytes do not fall below the normal range. A further striking phenomenon that has been regularly observed in CKD patients with PRCA is a marked and rapid increase in transferrin saturation and ferritin levels (Figure 2). In a series of 33 patients, median transferrin saturation was 80% at the time of diagnosis (18).

This also reflects the cessation of erythropoiesis and, thus, the interruption of iron utilization. In some instances, skin reactions have been observed at the sites of recent or former subcutaneous injections, which points to an important role of local immune reactions in the pathogenesis of anti-EPO antibody formation (22).

Definite diagnosis of epoetin-induced PRCA requires two confirmatory investigations: a bone marrow examination and the demonstration of anti-EPO antibodies in the patient's serum. Both investigations should be performed when the diagnosis is suspected. Cases in which the results of either the antibody test or the bone marrow examination are not available or inconclusive should, however, not be excluded from epidemiologic surveys and need to be reported to health authorities. In fact, databases maintained by manufacturers of epoetins contain a significant percentage of cases for which the diagnostic work-up and evidence for epoetin-induced PRCA is incomplete. A possible case definition that takes into account the different levels of certainty in the diagnosis is proposed in Table 3.

Assays for Anti-EPO Antibodies

Since epoetin-induced PRCA is due to the production of neutralizing anti-EPO antibodies, the identification of these antibodies in serum samples is the key to the diagnosis. Four different types of tests have been developed that have different advantages and limitations. The characteristics of individual assays in each category may vary depending on protocol, format, and reagents.

A radioimmunoassay (RIA) was used in the initial paper that described the upsurge of cases of epoetin-induced PRCA (8). Briefly, the serum samples are incubated first with ^{125}I -radiolabeled epoetin and then with protein G, which binds IgG. The antibody/protein G complexes are captured by centrifugation and the radioactivity of the pellet is measured (Figure 4). This test is very sensitive because it can detect the presence of high-affinity anti-EPO IgG when their concentration is as low as 10 ng/ml (S. Swanson; personal communication, September 2003). Furthermore, follow-up of 29 patients who recovered from epoetin-induced PRCA shows

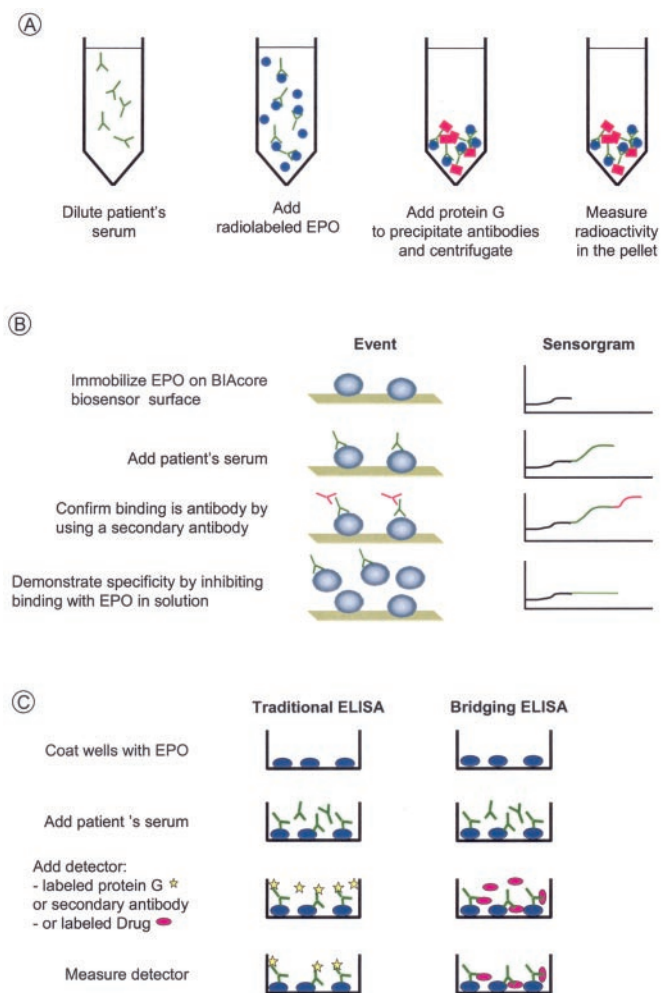


Figure 4. Schematic presentation of the 3 antibody tests that have been developed for testing sera for the presence of anti-EPO antibodies. (A) Radioimmunoassay (RIA). (B) Biosensor immunoassay. (C) Enzyme linked immunosorbent assay (ELISA).

that no patient had persistent signs of PRCA after the RIPA had become negative. This assay also appears to be highly specific and analysis of sera from 1340 CKD patients who were treated

Table 3. Proposed case definitions of suspected or proven anti-EPO antibody induced PRCA in patients treated with epoetin

	Loss of Efficacy of Epoetin ^a	Reticulocytes < 10,000/mm ³	Bone Marrow Examination Showing < 5% Erythroblasts ^b	Demonstration of Anti-EPO Antibodies
Clinically suspected case	Yes	Yes	n.d. ^c	n.d.
Suspected case with bone marrow confirmed PRCA	Yes	Yes	Yes	n.d.
Suspected case with anti-EPO antibodies	Yes	Yes	n.d.	Yes
Proven case of anti-EPO antibody induced PRCA	Yes	Yes	Yes	Yes

^a Treatment with epoetin for at least several weeks, plus Hb ↓ of approximately 1 g/dl per wk while receiving a constant or increased dose of epoetin or requirement for RBC transfusions.

^b In otherwise normal marrow.

^c n.d., not documented.

with epoetin, but who had no symptoms of PRCA, gave a negative result in all but four cases (23). Three subjects (0.2%) had borderline levels of anti-EPO antibodies. The fourth one, who had a weakly positive test, was a patient with a history of epoetin-induced PRCA. He had been treated with low dose cyclosporine and then rechallenged with epoetin alfa. Limitations of RIPA are that it cannot detect anti-EPO IgM and that it may not be able to detect the presence of low affinity antibodies. Thus, there is a theoretical possibility to obtain false negative results at a very early stage of the disease.

The biosensor immunoassay (BIAcore assay) is a sophisticated assay platform (24) that has recently been applied to the detection of EPO antibodies (25). Briefly, epoetin is immobilized on the surface of a sensorchip, and this biosensor is incubated with serum samples. Binding of anti-EPO antibodies is detected in real time according to mass accumulation on the biosensor surface (Figure 4). This test allows not only the detection of the presence of anti-EPO antibodies, but also the measurement of their binding affinities. The isotype of the antibodies can also be determined by adding a second antibody over the sensorchip. It is less sensitive than RIPA, with a sensitivity limit of about 100 ng/ml (S. Swanson; personal communication, September 2003), but it can detect low-affinity antibodies. This probably explains that the test gave a positive result in 59 out of 1500 serum samples from asymptomatic CKD patients (S. Swanson, unpublished data). Among these 59 patients, 21 had no documented prior exposure to epoetin. All responded to treatment with darbepoetin alfa, and analysis of a subsequent serum sample showed stable concentrations of anti-EPO antibodies in 49 subjects and no antibodies in 10. It is possible that some of these antibodies correspond to natural nonpathogenic anti-EPO antibodies. The major disadvantage of the biosensor assay is undoubtedly the price of the corresponding equipment (BIAcore), which is so far only available in a few high-tech laboratories.

Enzyme linked immunosorbant assays (ELISA) are quantitative methods that are widely used to detect antibodies, mostly because they are easy to implement. EPO is bound to multiwell plates and the binding of antibodies in patient serum is detected with the use of secondary antibodies (Figure 4). Sensitivity and specificity are very much dependent on the individual assay. Depending on the washing conditions, the specificity may be low (nonspecific binding of antibodies to the plate) or the sensitivity may decrease (low affinity antibodies being washed off). Increased specificity can be achieved by adding a second step, testing the displacement of bound secondary antibody by excess EPO. To further increase specificity, so-called bridging ELISAs have been developed. In bridging ELISAs, the two arms of anti-EPO antibodies form a bridge between epoetin immobilized to the surface of a microtiter plate and labeled epoetin that can be detected using a colorimetric assay. The microtiter plates coated with epoetin are thus incubated first with serum samples and then with labeled epoetin, which is detected using a colorimetric assay (Figure 4). Amgen developed a bridging ELISA with a sensitivity of 600 ng/ml that failed to detect antibodies in two subjects that had anti-epoetin antibodies at a concentration

higher than 2 mg/ml (S. Swanson; personal communication, September 2003). A bridging ELISA developed by Roche was reported to show a high concordance with RIPA results (A. Haselbeck; personal communication, September 2003). However, analysis of weakly positive samples suggests that this test may still have inferior sensitivity as compared to the RIPA (N. Casadevall, unpublished results) and more extensive comparisons are required.

Based on the data summarized above, the RIPA currently appears to be the best available assay for detection of anti-EPO antibodies that have caused PRCA. When this assay becomes more widely used, it will be crucial not only to precisely standardize it, but also to ensure that it is implemented in a limited number of reference laboratories, in which confirmatory tests can be performed. This will be important not only for individual patient care, but also for obtaining reliable epidemiologic data regarding this rare disease.

While the tests described above can detect the presence of anti-EPO antibodies, they cannot assess their neutralizing capability. *In vitro* bioassays, the fourth assay category, are the only tests that can demonstrate the ability of antibodies to neutralize endogenous EPO. They are based on the ability of the patient's serum (or ideally of the patient's immunoglobulins) to inhibit the growth of RBC precursors obtained from the bone marrow of healthy donors or of a cell line that depends on EPO for proliferation. So far, analysis in 50 cases of epoetin-induced PRCA has shown that the anti-EPO antibodies were in all cases able to neutralize the biologic effects of EPO (N. Casadevall, unpublished data). Therefore, given the expenditure associated with these bioassays, they do not appear to be indispensable for the diagnosis of epoetin-induced PRCA, and should probably only be performed in cases in which the results obtained with other tests are inconsistent with the clinical presentation.

Characterization of Anti-EPO Antibodies

Analysis of the anti-EPO antibodies detected in 13 patients with epoetin-induced PRCA revealed that they recognized the protein moiety of the molecule because they bound native epoetin as well as deglycosylated epoetin (8). The epitope that is recognized by the antibodies seems to be almost always conformational and denaturation of the protein completely abolished the binding of the antibodies in all but one of these 13 cases. In this latter case, the patient appeared to have produced antibodies against both a linear and a conformational epitope. The precise mapping of the epitope has not yet been successfully performed because of its conformational nature. Analysis of 8 patients with epoetin-induced PRCA has shown that, in 6 cases, anti-EPO IgGs were mostly IgG4, while in the two remaining cases, they were mainly IgG1 (S. Swanson; personal communication, September 2003). The existence of an IgM to IgG switch in the antibody response points to a role of T cells, which is also supported by *in vitro* studies (26). However, analysis of T cells from patients with epoetin-induced PRCA has yet to be done.

It is interesting to note that many features observed in patients with anti-EPO antibodies are reminiscent of what is

observed in patients with mild to moderate hemophilia A who develop anti-factor VIII antibodies. Development of such antibodies is rare in these patients. However, when it occurs, the antibodies frequently recognize both normal factor VIII and the patient's mutated own factor VIII. Furthermore, they are mostly of the IgG4 isotype, they recognize conformational epitopes, and they have neutralizing activity. Interestingly, analyses of the T cell response in patients with anti-factor VIII antibodies could only show minor differences between factor VIII-specific T cells from healthy individuals and from hemophilia A patients with or without inhibitor (27). Furthermore, despite extensive studies, predisposing factors for the development of anti-factor VIII antibodies remain mostly unknown. Besides the fact that these antibodies occur more frequently in patients with severe hemophilia A, only a weak link with an HLA haplotype (DRB1*1501, DQB1*0602, DQA1*0102) has been demonstrated (28).

Epidemiology of Anti-EPO Antibody-Induced PRCA

During the first 10 yr after rHuEPO became available for treatment of patients with CKD-related anemia, only 3 cases of epoetin-induced PRCA were described, while several million patients received treatment (5–7). Thus, the possibility for epoetin to induce the formation of anti-EPO antibodies was considered extremely low. However, since 1998, a sudden and large increase in the number of cases of epoetin-induced PRCA has been observed among patients with CKD.

The majority of cases have occurred in patients treated with epoetin alfa produced by Ortho-Biotech and marketed outside the United States (*i.e.* in patients treated with Eprex®/Erypo®) (10). Data made available by this pharmaceutical company indicate that, between January 1998 and July 2003, 184 cases of antibody-mediated PRCA occurred in patients exposed to Eprex® either alone (169 cases) or in association with another epoetin (15 cases), and that 62 cases are still being investigated (29). Out of those cases, not one has been confirmed in which epoetin alfa was exclusively administered intravenously (J. Knight; personal communication, September 2003). For the same time period, Roche, the manufacturer of epoetin beta, reports 8 cases attributable to NeoRecormon (cut-off June 30; E. Fröhlich; personal communication, October 2003). Since July 1997, 5 cases of antibody-mediated PRCA have been observed with epoetin alfa produced by Amgen and marketed in the United States either by Amgen (Epogen®) or by Johnson & Johnson (Procrit®). Finally, Amgen has not reported any case in patients solely exposed to darbepoetin alfa. Thus, to date, the total number of cases of epoetin-induced PRCA is probably close to 250.

Surprisingly, analysis of the annual incidence of epoetin-induced PRCA per country shows significant differences among different European countries. The highest incidence for PRCA induced by epoetin alfa has been observed in France and United Kingdom (J. Knight; personal communication, September 2003). For example, since 1998, 34 cases of epoetin-induced PRCA have been reported among CKD patients in France and 8 in Germany, including 24 and 7 patients on dialysis, respectively. The total number of patients on dialysis

in France and Germany is about 28,000 and 57,000, respectively. Thus, if one only considers patients on dialysis, the average annual incidence of epoetin-induced PRCA since 1998 has been around 1.7 cases per 10,000 patients in France and 0.26 cases per 10,000 patients in Germany. The reasons for this difference are unclear. It cannot be explained by differences in market share between the different brands of epoetin. The different European countries receive epoetins that come from the very same factories, and thus there cannot be any difference in the manufacturing processes. Differences in the route of administration of epoetin exist, but they can only explain a minor part of the difference observed between Germany and France, even though Germany and Austria have been the two European countries with the highest proportion of dialysis patients treated with intravenous epoetin. Thus, it is likely the difference in storage and handling account for the difference in incidence of epoetin-induced PRCA. The existence of few examples of temporal and spatial clustering of PRCA cases fits with this hypothesis.

Analysis of the total number of cases of epoetin alfa-induced PRCA shows that it progressively increased to peak in 2001 and 2002 (29). Similarly, in Europe, the annual incidence of epoetin-induced PRCA among CKD patients receiving Eprex® peaked in the second half of 2001 and it has been sharply decreasing since then (29). It went from about 4.5 cases per 10,000 patients in the second half of 2001 to 2.7 during the first half of 2002, 2.1 during the second half of 2002, and 0.5 during the first half of 2003 (Figure 5). Analysis of all cases of epoetin-induced PRCA that were reported in France and Germany since 1998 also shows a similar trend. The number of cases was highest in the second half of 2001 and first half of 2002, and only one case has been reported in 2003.

Interestingly, so far no case of epoetin-induced PRCA has been reported in cancer patients on chemotherapy, although the anemia of malignant disease has become a frequent indication for epoetin therapy. Only two cases have been observed in patients not treated for CKD-related anemia. Both were French patients with myelodysplastic syndrome. One was receiving

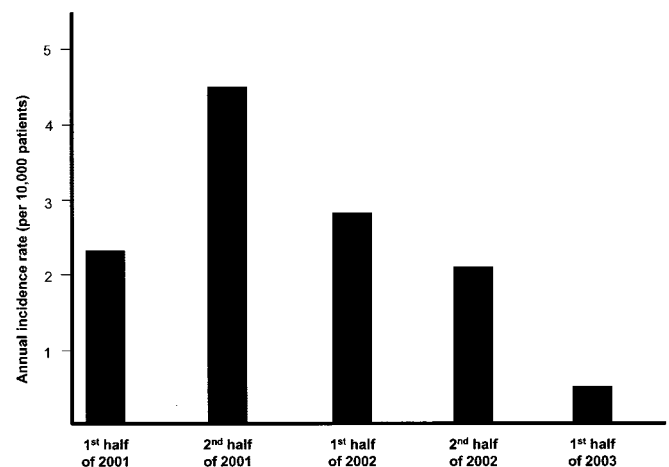


Figure 5. Incidence rates of PRCA cases observed in Europe in association with the use of epoetin alfa (29).

epoetin alfa and the other one epoetin beta (N. Casadevall, S. Giraudier, L. Quint, unpublished data). Potential reasons for this apparent protection of cancer patients include shorter duration of therapy, nonspecific immunosuppression, or as yet unidentified differences in drug handling and exposure. It is noteworthy, for example, that prefilled syringes with different doses are usually used for CKD patients and cancer patients.

Analysis of the route of administration of epoetin in patients who developed PRCA indicated early that the vast majority, if not all, were receiving epoetin subcutaneously. Thus, at the end of 2002, health authorities decided to contraindicate the subcutaneous injection of epoetin alfa in Europe, and it was strongly discouraged in Canada and Australia. This induced a practice change, which was rapidly followed by a sharp decrease in the number of cases of epoetin-induced PRCA, as outlined above. Although additional measures were implemented at the same time, including increased attention to storage and handling, the decline in incidence seems to confirm that subcutaneous administration of epoetin alfa is a strong risk factor for the development of this complication. On the other hand, the lack of a close temporal relationship between the upsurge of epoetin-induced PRCA and the increased subcutaneous use of epoetin, as well as the association with primarily one brand of epoetin, clearly indicate that additional factors must have played an important role.

Possible Causes and Risk Factors of Immunogenicity of Epoetins

Although biopharmaceuticals are designed as copies of naturally occurring molecules, their immunogenicity is a well-recognized problem. There is probably not a single recombinant molecule used in clinical medicine that has not been found to induce antibody formation in at least some cases (30). The consequences of such antibodies vary widely. While many are clinically insignificant, others can reduce or even increase the efficacy of the recombinant molecules, and some—as in the case of epoetin—cross-react with the endogenous protein and inhibit its effects.

In general, two different mechanisms of antibody formation have to be distinguished. In individuals who completely fail to produce a certain protein (*e.g.* a coagulation factor), recombinant molecules can be recognized as foreign and thus elicit a “physiologic” immune response. In those cases where the recombinant product is used to substitute for deficient production of an endogenously produced molecule, as in the case of epoetins, the pathophysiology is more complex and reflects a breakdown of self-tolerance. In general, such an immunogenic reaction can be triggered by a variety of factors, including variations of the protein sequence, differences in glycosylation, protein aggregation, contaminants, and impurities occurring during the production process, as well as components and properties of the formulation (30). In addition, factors such as individual predisposition, patient immune status, route of administration, and length of treatment play an important role.

The observation that subcutaneous administration of epoetin is an important risk factor is in line with the fact that intravenous use of proteins is generally associated with the lowest risk

of immunogenicity (30). Regarding the association with the formulation of epoetin alfa marketed outside the United States, there is no reason to believe that the amino acid sequence of the molecule is different in this particular brand. Subtle differences exist between the carbohydrate moieties of epoetin alfa and beta (31,32), but it seems unlikely that they have significant impact on immunogenicity. Moreover, there is no indication that the glycosylation pattern of epoetin alfa has changed over time and could therefore explain the upsurge of PRCA cases.

The important clue apparently comes from the temporal coincidence with a change in the formulation of epoetin alfa sold outside the United States. Upon request from the European Agency for the Evaluation of Medicinal Products (EMA; London, UK) the manufacturer of Eprex®/Erypo® removed human serum albumin (HSA) from the formulation, and replaced it with Tween 80 (polysorbate 80) to avoid potential contamination by HIV and Creutzfeldt-Jacob disease-causing prions. An exception is the sale of multiusage vials in Canada, that still contain HSA. The formulation of epoetin beta has always been HSA-free, but the stabilizer composition differs from that of HSA-free epoetin alfa (Table 4). It has been postulated that this change in formulation might have reduced the stability of the formulation, allowing the formation of aggregates; in particular, when handling instructions, such as cold storage, were not followed. Contamination with silicone, used to lubricate prefilled syringes, has also been considered as an additional risk factor. However, neither has increased formation of aggregates been demonstrated so far in vials of epoetin alfa, nor has definite proof been provided that such aggregates could lead to a critical increase in immunogenicity.

Recently, Schellekens *et al.* suggested another explanation. They propose that the concentration of Tween 80 in the new formulation of epoetin alfa is so high that it leads to micelle formation and that epoetin molecules are integrated into the surface of these micelles. As a consequence, several epoetin molecules are presented to the recipient’s immune system in a regular spatial configuration, which can trigger the immune system (33). Similar phenomena have been recognized in the immune recognition of viruses, where antigens integrated into the virus wall at regular distance appear to play an important role (34).

It also remains possible that a contaminant present in the end product could act as an immunological adjuvant. In fact, recent investigations focus on potential release of organic compounds from the rubber plungers of prefilled syringes by the action of the detergent polysorbate 80. These rubber stoppers were only used for epoetin alfa syringes marketed outside the US by Ortho-Biotech (Eprex®/Erypo®), and they have meanwhile been replaced by teflon-coated plungers (J Knight; personal communication, September 2003).

To date, no patient-related risk factors have been identified that might have an impact on the development of epoetin-induced PRCA. Most patients who developed epoetin-induced PRCA did not have a history of autoimmune disease or of drug-induced immune reaction. Genotyping of 10 patients did not reveal any variation in the sequence of the endogenous EPO protein, which could have explained why the recombinant

Table 4. Different formulations of epoetin alfa and beta marketed in different parts of the world

	Epoetin Alfa				Epoetin Beta	
	US	Canada	Europe	Australia	Europe	
	Epogen, Procrit	Eprex multiuse vials	Eprex single-use syringes	Eprex	Eprex	NeoRecormon
Human albumin	Yes	Yes				
Sodium chloride	Yes	Yes	Yes	Yes	Yes	Yes
Calcium chloride						Yes
Sodium citrate	Yes	Yes			Yes	
Citric acid	Yes	Yes				
Sodium phosphate	Yes ^a		Yes	Yes	Yes	
Urea						Yes
Polysorbate 80			Yes	Yes	Yes	
Polysorbate 20						Yes
Glycine			Yes	Yes	Yes	Yes
Leucine						Yes
Isoleucine						Yes
Threonine						Yes
Glutamic acid						Yes
Phenylalanine						Yes
Other	Benzyl alcohol ^b	Benzyl alcohol				

^a Sodium phosphate only included in multidose vials containing 40,000 IU/ml.

^b Preservative free vials also available.

molecule was considered as foreign. There also was no obvious association with particular HLA haplotypes in a limited number of investigated cases (P. Mayeux and N. Casadevall, unpublished data).

Natural Course and Response to Therapy

Obviously, once the diagnosis of epoetin-induced PRCA is suspected or has been proven, epoetin therapy needs to be discontinued. As far as investigated, anti-EPO antibodies crossreact not only with the endogenous hormone, but also with all recombinant EPO molecules, including darbepoetin alfa. Therefore, switching the brand of epoetin does not improve the anemia and obscures the causality. Moreover, continued exposure to epoetin implies the risk of severe systemic immune reactions (22).

On the other hand, available data suggest that cessation of epoetin exposure alone is usually insufficient to induce recovery from epoetin-induced PRCA. Retrospective analysis of 45 cases of epoetin-induced PRCA showed that eight patients did not receive any specific treatment, besides stopping the administration of epoetin (18). All eight patients still had PRCA after a median follow-up of 12 mo. In contrast, administration of immunosuppressive therapy appears to greatly enhance the likelihood of recovery. Out of 36 patients who received some immunosuppressive therapy in addition to stopping epoetin administration, anti-EPO antibodies disappeared and reticulocyte counts consistently rose above 10,000/mm³ in 78% of cases (18). However, defining the optimal therapy is difficult. Analysis of the 36 aforementioned cases shows that recovery was observed in 11 of 19 cases treated with corticosteroids

(58%), in six of eight cases who received corticosteroids plus cyclophosphamide (75%), in 4 of 6 cases treated with cyclosporine (67%), and in all six patients who had a kidney transplant. Three additional patients have been reported who rapidly recovered from epoetin-induced PRCA after receiving a kidney transplant (35). In contrast, treatment with high doses of immunoglobulins was successful in only one case of nine. Median recovery time was 4.3 mo for patients treated with corticosteroids alone, 3 mo for those treated with corticosteroids plus cyclophosphamide, only 1 mo for those treated with cyclosporine, and less than a month for those who received a kidney transplant. Whether the rapid recovery after transplantation is due to strong immunosuppression or is related to exposure to natural EPO produced by the transplant is yet unclear. So far, no relapse of PRCA has been reported after stopping immunosuppressive therapy. Whether epoetin treatment can be safely resumed at some stage is unclear, but is a crucial question for patients who have recovered from epoetin-induced PRCA.

Conclusions, Open Questions, and Future Developments

A review of this issue can naturally provide only a snap shot picture of a rapidly developing field. Any conclusion at this stage, therefore, has to be considered as somewhat preliminary. With due recognition of this limitation, it appears as if the initial concerns that the reported cases reflect only the tip of an iceberg fortunately do not turn out to be true. In fact, given the typical clinical presentation and the publicity that this complication has achieved in the context of a highly competitive and

profitable market, one might assume that the number of unrecognized and unreported cases is probably not very high. Nevertheless, a variety of important questions remain to be answered and diagnosis and follow-up of cases definitely need to be improved.

To this end assays for anti-EPO antibodies need to be standardized and reference laboratories should be established. At the same time, agreement has to be achieved about case definitions and an independent case registry would be desirable. Anti-EPO antibodies need to be further characterized and their properties carefully compared with clinical symptoms to assess the relevance of different concentrations, subtypes and affinities. Analysis of T cell response in patients with epoetin-induced PRCA should also be undertaken in parallel. Prospective epidemiologic studies to establish the background prevalence of anti-EPO antibodies, in patients treated and not treated with epoetins, have only just started and need to be continued. So far, the available evidence indicates that anti-EPO antibodies do not play a relevant role as causes of EPO-resistance in patients who do not present with the typical symptoms of PRCA. However, this conclusion also needs to be confirmed in larger studies.

The optimal management of individual cases is far from being clear. Observational follow-up is currently the only source of information; and depending on the development of incidence rates, prospective treatment trials comparing the most promising therapeutic options should be considered. In those patients who have recovered from epoetin-induced PRCA, the question arises whether and under which conditions they can be re-exposed to epoetin.

Finally, the recent experience with anti-EPO antibodies may also have considerable implications for the future approval of epoetin preparations and other biopharmaceuticals. Irrespective of the fact that the genetic code precisely defines the amino acid sequence of recombinant molecules, the impact of difference in the formulation and production process on immunogenicity appears to be a real concern. In Europe, at least ten companies have applied for approval of a follow-on biologic version (“biogeneric”) of EPO (36). In view of the recent experience with a licensed product, health authorities face the question of how to specify safety requirements during phase III trials and postmarketing surveillance of these new drugs, without increasing the demands to a level that is prohibitive for their development.

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for comments and for confirmation of certain details and personal communications. However, the paper summarizes the issue to the best knowledge of the authors and does not necessarily reflect the position and knowledge of different companies.

References

- Garcia JF, Clemmons G: The radioimmunoassay of erythropoietin. In: *Recent Advances in Nuclear Medicine*, Vol. VI, edited by Lawrence JH, New York, Grune & Stratton, 1983: 19–40.
- Kaufman JS, Reda DJ, Fye CL, Goldfarb DS, Henderson WG, Kleinman JG, Vaamonde CA: Subcutaneous compared with intravenous epoetin in patients receiving hemodialysis. Department of Veterans Affairs Cooperative Study Group on Erythropoietin in Hemodialysis Patients. *N Engl J Med* 339: 578–83, 1998
- Anonymous: NKF-K/DOQI Clinical Practice Guidelines for Anemia of Chronic Kidney Disease: Update 2000. *Am J Kidney Dis* 37[Suppl 1]: S182–S238, 2001
- Anonymous: European Best Practice Guidelines for the Management of Anaemia in Patients with Chronic Renal Failure Guideline 9: Route of administration of epoetin. *Nephrol Dial Transplant* 14[Suppl 5]: 19–20, 1999
- Bergrem H, Danielson BG, Eckardt K-U, Kurtz A, Stridsberg M: A case of anti-erythropoietin antibodies following recombinant human erythropoietin treatment. In: *Erythropoietin: Molecular Physiology and Clinical Application*, edited by Bauer C, Koch KM, Scigalla P, New York, Marcel Dekker, 1993, pp 266–275
- Peces R, de la Torre M, Alcazar R, Urrea JM: Antibodies against recombinant human erythropoietin in a patient with erythropoietin-resistant anemia. *N Engl J Med* 335: 523–524, 1996
- Prabhakar SS, Muhlfelder T: Antibodies to recombinant human erythropoietin causing pure red cell aplasia. *Clin Nephrol* 47: 331–335, 1997
- Casadevall N, Nataf J, Viron B, Kolta A, Kiladjian JJ, Martin-Dupont P, Michaud P, Papo T, Ugo V, Teyssandier I, Varet B, Mayeux P: Pure red-cell aplasia and anti-erythropoietin antibodies in patients treated with recombinant erythropoietin. *N Engl J Med* 346: 469–475, 2002
- Eckardt KU, Casadevall N: Pure red-cell aplasia due to anti-erythropoietin antibodies. *Nephrol Dial Transplant* 18: 865–869, 2003
- Gershon SK, Luksenburg H, Cote TR, Braun MM: Pure red-cell aplasia and recombinant erythropoietin. *N Engl J Med* 346: 469–475, 2002
- Krantz SB: Pure red-cell aplasia. *N Engl J Med* 291: 345–350, 1974
- Erslev AJ: Pure red cell apalsia. In: *Williams Hematology*, 6th ed., edited by Beutler E, Lichtman MA, Coller BS, Kipps TJ, Seligsohn U, New York, McGraw-Hill, 2000, pp 391–398
- Peschle C, Marmont AM, Marone G, Genovese A, Sasso GF, Condorelli M: Pure red cell aplasia: Studies on an IgG serum inhibitor neutralizing erythropoietin. *Br J Haematol* 30: 411–417, 1975
- Casadevall N, Dupuy E, Molho-Sabatier P, Tobelem G, Varet B, Mayeux P: Autoantibodies against erythropoietin in a patient with pure red-cell aplasia. *N Engl J Med* 334: 630–633, 1996
- Linardaki GD, Boki KA, Fertakis A, Tzioufas AG: Pure red cell aplasia as presentation of systemic lupus erythematosus: Antibodies to erythropoietin. *Scand J Rheumatol* 28: 189–191, 1999
- Geetha D, Zachary JB, Baldado HM, Kronz JD, Kraus ES: Pure red cell aplasia caused by Parvovirus B19 infection in solid organ transplant recipients: A case report and review of literature. *Clin Transplant* 14: 586–591, 2000
- Brown KE, Young NS: Parvovirus B19 in human disease. *Annu Rev Med* 48: 59–67, 1997

18. Verhelst D, Rossert J, Casadevall N, MacDougall IC, Kruger A, Eckardt KU: Treatment of antibody-mediated pure red cell aplasia. *J Am Soc Nephrol* 14: 27A, 2003
19. Cavill I, Williams JD: Benefits of recombinant human erythropoietin. *Lancet* 360: 1606–1607, 2002
20. Ishibashi T, Koziol JA, Burstein SA: Human recombinant erythropoietin promotes differentiation of murine megakaryocytes in vitro. *J Clin Invest* 79: 286–289, 1987
21. Fraser JK, Tan AS, Lin FK, Berridge MV: Expression of specific high-affinity binding sites for erythropoietin on rat and mouse megakaryocytes. *Exp Hematol* 17: 10–16, 1989
22. Weber G, Gross J, Kromminga A, Loew HH, Eckardt KU: 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: 2381–2383, 2002
23. Wu G, Nathoo B, Mendelssohn D, Pandeya S: Antibody to erythropoietin in surveillance study in 5 Ontario renal centers. *Nephrol Dial Transplant* 18[Suppl 4]: 162, 2003
24. Van Regenmorte: Improving the quality of BIACORE-based affinity measurements. *Dev Biol* 112: 141–151, 2003.
25. Mason S, La S, Mytych D, Swanson SJ, Ferbas J: Validation of the BIACORE 3000 platform for detection of antibodies against erythropoietic agents in human serum samples. *Curr Med Res Opin* 7: 651–659, 2003
26. Netti GS, Petruzzelli V, Blasi A, Roca L, Ranieri E, Di Paolo S, Pertosa G, Grandaliano G, Schena FP, Gesualdo L: May recombinant erythropoietin induce a specific T-cell-mediated immune response. *Nephrol Dial Transplant* 18[Suppl 4]: 161, 2003
27. Reding MT, Wu H, Krampf M, Okita DK, Diethelm-Okita BM, Christie BA, Key NS, Conti-Fine BM: Sensitization of CD4+ T cells to coagulation factor VIII: Response in congenital and acquired hemophilia patients and in healthy subjects. *Thromb Haemost* 84: 643–652, 2000
28. Hay CR, Ollier W, Pepper L, Cumming A, Keeney S, Goodeve AC, Colvin BT, Hill FG, Preston FE, Peake IR: HLA class II profile: A weak determinant of factor VIII inhibitor development in severe haemophilia A. UKHCDO Inhibitor Working Party. *Thromb Haemost* 77: 234–237, 1997
29. http://www.jnj.com/news/jnj_news/1021024_095632.htm?textOnly=false. Accessed November, 2003.
30. Schellekens H: Bioequivalence and the immunogenicity of biopharmaceuticals. *Nat Rev Drug Discov* 1: 457–462, 2002
31. Storring PL, Tiplady RJ, Gaines Das RE, Stenning BE, Lamiakanra A, Rafferty B, Lee J: Epoetin alfa and beta differ in their erythropoietin isoform compositions and biological properties. *Br J Haematol* 100: 79–89, 1998
32. Skibeli V, Nissen-Lie G, Torjesen P: Sugar profiling proves that human serum erythropoietin differs from recombinant human erythropoietin. *Blood* 98: 3626–3634, 2001
33. Hermeling S, Schellekens H, Daan J. A, Crommelin, Jiskoot W: Micelle-associated protein in epoetin formulations: A risk factor for immunogenicity? *Pharm Res* 20: 1903–1908, 2003
34. Bachmann MF, Hoffmann RU, Kündig TM, Bürki K, Hengartner H, Zinkernagel RM: The influence of antigen organization on B cell responsiveness. *Science* 262: 1448–1451, 1993
35. Chng WJ, Tan LK, Liu TC: Cyclosporine treatment for patients with CRF who developed pure red blood cell aplasia following EPO therapy. *Am J Kidney Dis* 41: 692–695, 2003
36. Louet S: Lessons from Eprex for biogeneric firms. *Nat Biotechnol* 21: 956–957, 2003

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