Antibody-mediated pure red cell aplasia in chronic kidney disease patients receiving erythropoiesis-stimulating agents: new insights

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Antibody-mediated pure red cell aplasia is a very rare but devastating condition affecting patients receiving treatment with erythropoiesis-stimulating agents. New cases continue to emerge, generally in clusters, consistent with an 'environmental' trigger to its pathogenesis. Defining the causes of antibody-mediated pure red cell aplasia is clearly of importance for patients with chronic kidney disease, but any developments in this area may also have relevance to other disease areas as therapeutic delivery of endogenous proteins rapidly increases. This review focuses on the current knowledge regarding the etiology of antibody-mediated pure red cell aplasia and the current approach to therapy.

Kidney International (2012) **81,** 727–732; doi:10.1038/ki.2011.500; published online 15 February 2012

KEYWORDS: anemia; chronic kidney disease; clinical nephrology; pure red cell aplasia

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Received 30 June 2011; revised 13 September 2011; accepted 27 September 2011; published online 15 February 2012

Antibody-mediated pure red cell aplasia (PRCA) is characterized by a sudden fall in hemoglobin concentration despite erythropoiesis-stimulating agent (ESA) therapy, with reticulocyte counts declining to levels $<20 \times 10^9$/l. Affected patients rapidly become transfusion dependent, and a bone marrow examination shows absence or near-absence of erythroid progenitor cells. Antibodies to erythropoietin (EPO), detectable in the serum of such patients, neutralize not only the biological activity of the therapeutic ESA, but also endogenous EPO, thus obliterating red cell production in the bone marrow.

PRCA related to ESA therapy is rare, with an exposure-adjusted incidence of 0.02–0.03 per 10,000 patient-years.[1] The peak incidence of PRCA related to ESA therapy occurred during 2002–2003, following the report of a small case series.[2] The majority of these cases were caused by a preparation of epoetin alfa marketed outside the United States (Eprex/Erypo). During this time, the exposure-adjusted incidence rate per 10,000 patient-years for Eprex-associated PRCA peaked at 4.5.[1] The cause of this condition has remained elusive, although several factors are believed to have been implicated. The most obvious cause was the removal of human serum albumin (HSA) from the Eprex preparation, which was mandated by European law following concerns about the transmission of Creutzfeldt-Jakob disease. HSA was replaced by polysorbate 80, and it was initially thought that the polysorbate itself might be involved in the pathogenesis. The 'mixed micelle' hypothesis has, however, been found to be less attractive than was initially thought, and the 'rubber leachates' hypothesis has also been challenged. Another factor in the pathogenesis of this condition was believed to be a break in the cold storage chain, thus rendering the protein molecule less stable.

Despite these explanations, there are still unanswered questions. For example, cases of PRCA were not seen exclusively in patients receiving HSA-free epoetin alfa, but also in

patients receiving epoetin beta and darbepoetin alfa. Also, if the 'rubber leachates' hypothesis was correct, why did only a very small fraction of the patients develop this condition? Following a flurry of literature in the period 2002–2007, the scientific world has gone quiet regarding this condition. However, new cases continue to develop across Europe and elsewhere.[3,4] At the time of writing, five new cases have been detected in the United Kingdom alone over the last 6 months, a case of antibody-mediated PRCA in a patient treated exclusively with intravenous epoetins has been reported,[5] and the first case in a patient receiving methoxy polyethylene glycol–epoetin beta (Mircera) has emerged.[6] In addition, one definite case of PRCA and one possible case have emerged during a clinical trial of a biosimilar EPO involving only 337 patients.[7] This trial was aborted, and the manufacturer of this biosimilar EPO embarked on an extensive investigation into the possible root cause of this problem.[8]

ROOT CAUSES OF INCREASED IMMUNOGENICITY OF ESAs

Product-related factors

Following the increase in PRCA associated with Eprex/Erypo, the manufacturer initiated a program of investigations to identify possible causes. However, the question of exactly how the change in formulation of this product led to increased immunogenicity has not been fully resolved. The answer is important not only for the safety evaluation of future ESAs, but also for the development of therapeutic proteins in general.

As epoetins, like many other biopharmaceuticals, are recombinant versions of human proteins, the issue is one of autoimmunity and more specifically the breaking of B-cell tolerance. The mechanisms by which tolerance is broken are not completely understood. Many factors have been reported to influence immunogenicity (Figure 1); the primary factor responsible for activation of autoreactive B cells, supported by clinical and experimental data, is the presence of aggregates.[9,10] The hypothesis is that the periodicity of self-antigens present in protein aggregates resembles the repeated self-epitope structure of viral capsids that is capable of directly activating B cells.

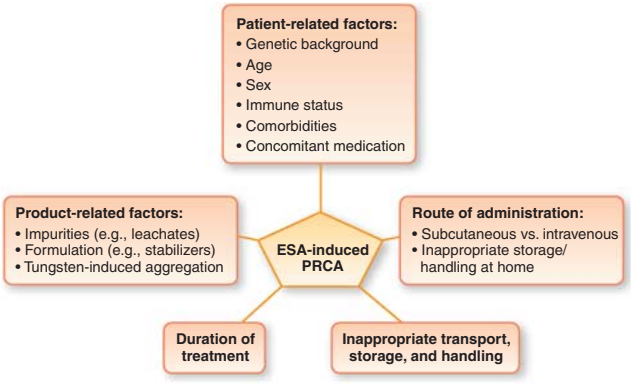

Figure 1 | Factors involved in the pathogenesis of erythropoiesis-stimulating agent (ESA)–induced pure red cell aplasia (PRCA).

Several explanations have been offered to explain how the exchange of HSA with polysorbate 80 led to an increase in the incidence of PRCA associated with Eprex/Erypo. Any plausible explanation should be based on experimental data and have a sound biological rationale. It should also be consistent with epidemiological data such as the rarity and uneven geographic distribution of PRCA. One explanation involved organic leachates from the uncoated rubber stoppers of pre-filled syringes, which were proposed to act as adjuvants for T-cell–mediated activation of the anti-EPO immune response. There are several reasons why this explanation is unlikely. First, the level of self-tolerance to EPO is very high, because an autoimmune response to this self-protein would be lethal; indeed, there is only one report of a case of PRCA caused by spontaneous production of antibodies.[11] Second, the leachates failed to show any effect on the immunogenicity of Eprex/Erypo in a mouse model;[12] given the high degree of homology between the amino-acid sequences of human and murine EPO, this lack of effect argues against the involvement of leachates in breaking B-cell tolerance. Leachates did yield a positive, concentration-dependent antibody response in the same model immunized with ovalbumin; however, the appropriateness of this model is questionable, given that ovalbumin is a foreign protein. The antibody response in this system is more likely a classical immune reaction to a foreign protein, not a loss of self-tolerance. The leachates hypothesis is also inconsistent with the epidemiological data; regional differences in incidence are difficult to explain with leachate adjuvants being present in every syringe. There was also no discussion of the cumulative effect of repeated exposure to low concentrations of leachates, and no explanation has been put forward for the mechanism by which leachates could behave as adjuvants.

A second explanation involves the formation of polysorbate 80 micelles containing protein, which were proposed to break tolerance by presenting epoetin to B cells in an array form.[13] However, their low concentration and instability make EPO-containing micelles a questionable mechanism for breaking B-cell tolerance. This hypothesis is also inconsistent with the epidemiological data because the micelles would also have been present in all syringes containing the reformulated product.

The theory based on aggregate formation, made more likely following the formulation change in Eprex/Erypo, is consistent with all available data and is supported by substantial experimental evidence from other therapeutic proteins.[14] Eprex/Erypo has been reported to contain increasing levels of aggregates with prolonged duration of storage, and the tendency for aggregate formation could be exacerbated by a high concentration of polysorbate 80.[15] A role for aggregation is also consistent with the epidemiological patterns. HSA was a more efficient stabilizer of epoetin than polysorbate 80; following the change of formulation, mishandling of less stable products would be more likely to induce aggregation. Divergent handling methods could

therefore explain the geographical distribution of ESA-associated PRCA. Later reports of immunogenicity problems with epoetins also support the proposal that aggregation induced by mishandling was the root cause of the spike in PRCA associated with Eprex/Erypo. Several cases of PRCA have been reported in Thailand associated with Eprex.[16] Mishandling resulting in aggregate formation was suggested as the reason for these cases.

There have also been cases of antibody-mediated PRCA reported following the use of locally manufactured epoetins in Latin America and Asia.[17,18] These alternative biological products (manufactured outside of Europe and the United States) should be distinguished from true biosimilar epoetins, which are approved under a strict regulatory pathway such as the one set out by the European Medicines Agency.[19–24] Biosimilar epoetins approved under the European Medicines Agency regulatory pathway are required to undergo extensive physicochemical characterization to confirm their similarity to the originator product.[25] The European Medicines Agency biosimilar regulatory pathway also requires clinical trial data in at least one representative indication and a comprehensive risk management plan.[19–22] The alternative biological products manufactured in Latin America and Asia typically required only bioequivalence data to gain marketing approval (as for conventional generic medicines), with no requirement for clinical trial data or formal pharmacovigilance.[18] To date, pharmacovigilance infrastructure in these territories has been lacking or inadequate, and therefore problems of immunogenicity are likely to have been underreported. It is important that such infrastructure is established to improve detection of adverse effects with biopharmaceuticals. In addition, analytical studies of these alternative products have shown that they vary in composition, indicating inadequate control over manufacturing processes.[26,27]

Following two cases of neutralizing antibodies (NAbs) in patients receiving subcutaneous HX575,[7] the manufacturer of this product initiated investigations to identify the possible root cause(s) of these adverse events. They have recently reported that pre-filled syringes containing HX575, implicated in two cases of NAbs, contained unexpectedly high levels of protein aggregates and tungsten. At least a proportion of the aggregates appeared to be dimers covalently linked by disulfide bonds.[8] Spiking of non-suspect batches of the medication with sodium polytungstate, or with an extract of tungsten pins used to manufacture the syringes, induced the formation of aggregates with similar properties; in addition, tungsten (sodium polytungstate) was shown to have a strong denaturing effect on the protein.[8] It is proposed that the tungsten species in the syringes caused HX575 protein to unfold, with subsequent formation of aggregates. This hypothesis is inherently attractive, as several other reports have also implicated tungsten in the aggregation of pharmaceutical protein products.[28–30] Further work to fully characterize these tungsten-induced dimers/aggregates is required to determine the epitopes responsible for immunogenicity. The tungsten is most likely to originate from tungsten pins used to form the barrels of the glass syringes in which the final product is provided for use.[30] As the syringes and needles are generally provided by a single supplier, this possibly explains why PRCA has been recognized with multiple ESA products. Furthermore, it is likely that the leaching of tungsten into the syringe occurs in a variable manner based on the life of the tungsten pin used. These findings may be more broadly applicable not only to other ESAs but also to other classes of therapeutic proteins. Given these novel findings, it may be prudent for manufacturers of therapeutic proteins to routinely determine the tungsten content of their syringes, and consider switching to alternative strategies to generate the barrels of their glass syringes in order to minimize the risk of protein aggregation and an immunogenic response.

Route of administration

In general, intravenous administration of a protein is much less likely to evoke an immune response than intranasal, intramuscular or subcutaneous administration.[31] For example, in one study the incidence of anti-interferon antibodies in patients treated subcutaneously was 10 times the rate among patients treated intravenously.[32] This may be due to the high concentration of antigen-presenting cells in the skin and/or the longer availability of the administered protein due to the slower rate of resorption.[33] This process is reflected in a case report of a patient with PRCA who repeatedly developed wheals at sites of former subcutaneous administration following intravenous administration of Eprex.[34] In addition, the subcutaneous route increases the possibility of self-administration and therefore inappropriate storage/handling at home.[35] It is clear that the majority of reported antibody-positive PRCA cases occurred in patients receiving epoetins by the subcutaneous route.[36] Two cases in which PRCA developed following intravenous administration of epoetin have been mentioned in the literature,[37] although a follow-up with the authors could not confirm that these cases involved intravenous use exclusively. A very recent report from Japan describes what appears to be the only confirmed report of PRCA arising solely from intravenous administration of epoetin, with the patient receiving both epoetin alfa and darbepoetin alfa on separate occasions.[5]

Duration of treatment

In the original case series reported by Casadevall *et al.*,[2] the interval from the start of therapy to EPO-refractory anemia ranged from 3 to 67 months. In a review of all cases of ESA-induced PRCA known up to April 2004, the median duration of treatment before PRCA was diagnosed was 9.1 months for patients receiving Eprex, 24.8 months for patients receiving Epogen, and 18.0 months for patients receiving NeoRecormon.[36] Sporadic cases reported in other indications also occurred after prolonged treatment.[38] A possible explanation for the longer median duration of treatment with Epogen is that this agent is used primarily in the United States, where it is predominantly administered intravenously, while the other

agents are used in Europe and elsewhere, with a greater proportion of subcutaneous administration. As described previously, intravenous administration of a protein is much less likely to evoke an immune response than subcutaneous administration.

However, these intervals may not be truly representative of the duration of treatment required for anti-EPO Abs to be generated. First, these data include PRCA cases from a period when a formulation change resulted in a product that was clearly more immunogenic than earlier and subsequent formulations. Second, nephrologists are now much more aware of PRCA, and are likely to more promptly initiate appropriate investigations in patients who exhibit a sudden decrease in blood hemoglobin levels and reticulocyte counts. Previously, it is possible that physicians were more likely to respond to such a situation by escalating the ESA dose or switching to an alternative product. In routine clinical practice, blood samples are tested for binding and NAbs only after resistance to ESA therapy is apparent. In reality, therefore, the period until antibodies form is likely to be shorter than previously indicated, and anti-EPO antibodies may be present after 3 months of therapy.

The duration of treatment has been proposed to explain, at least in part, why no cases of PRCA have been reported in patients with cancer receiving ESAs for chemotherapy-induced anemia, who typically receive ESA treatment for a much shorter period than patients with renal anemia. Such patients may also have a compromised immune response because of the myelosuppressive effects of chemotherapy.[35] It is also possible that PRCA has not been diagnosed in this population because of a low index of suspicion, with severe anemia attributed to marrow suppression by the chemotherapy itself or to the effects of the tumor.

Patient characteristics

As PRCA is a very rare and geographically and temporally disparate condition, with an often insidious onset, it is difficult to properly identify common themes and features. There may be only handfuls of accurately identified cases in any country at any one time, so it may be years before individual doctors or even teams of nephrologists recognize a case, or a series of cases.

It is apparent that the normal tolerance to foreign proteins (amino acids and sugar moieties) breaks down under selected, largely unpredictable, circumstances, to allow the production of specific NAbs. Under more normal conditions, there is immunological accommodation, if not true tolerance, to exogenous proteins, that is, 'broken' by clinical events—perhaps infection, intercurrent illness, or adjuvant therapies (e.g., tungsten or aggregates). This most likely relates to a complex and unpredictable interaction between the patient's immunological status (genotype and phenotype) and the protein in question, and additional factors such as the catalytic or tolerance-breaking effects referred to above. It is clearly a considerable co-incidental effort for the immunological accommodation to be overcome, and therefore almost impossible to screen for subjects who might be susceptible.

Several patient-related factors appear to be relevant, but may be of little or no use for screening. The genetic background of the patient can influence whether antibodies are produced;[39] the major histocompatibility complex allele affects antigen recognition in T-lymphocyte–mediated responses. Follow-up investigations into the cluster of antibody-positive PRCA cases reported in Thailand focused on HLA-DRB1*9, a major histocompatibility complex allele that is much more common in Thais than in Caucasians.[40] Thai patients who were carriers of this allele were more likely to have developed PRCA than a control group consisting of kidney transplant candidates,[41] and a similar finding has been reported in Caucasian patients.[42] However, cases also developed in patients who were not carriers of this allele. This suggests that while this major histocompatibility complex allele may contribute to the susceptibility to developing anti-EPO antibodies, it is not an absolute requirement.

It has been noted that there is an excess of elderly males among cases of ESA-related PRCA.[36] The two cases of NAbs with HX575 also occurred in elderly male patients.[7] However, as most cases occur outside of clinical trials, there is only limited information about the characteristics of the patients involved. An updated analysis would be of interest to establish if elderly males, or other patient groups, are at increased risk of PRCA secondary to ESA treatment.

CD4 T cells have a central role in the humoral immune response, and a recent study has quantified the number of EPO-specific CD4 T cells in the blood of normal donors.[43] These investigations identified an important repertoire of pre-existing EPO-specific T cells in almost half of the donors, comparable to that of non-self-proteins. The authors suggest that, at steady state, endogenous EPO contributes weakly to induction of tolerance and may be ignored by the immune system. Consequently, circulating EPO-specific CD4 T cells could be susceptible to activation by altered batches of exogenous EPO, providing them with co-stimulatory signals. These data also suggest that T-cell assays in normal donors may be useful in determining the immunogenic potential of therapeutic proteins.[43] This is interesting and potentially promising work, but overall the science of drug-induced immunological changes is not given the prominence, funding, and priority it deserves.

Diagnosis and treatment of ESA-induced PRCA

PRCA is a primary hematologic disease, but can also occur as a result of various infections, hematologic malignancies, autoimmune diseases, severe malnutrition, and exposure to certain drugs and toxins.[3] The first clinical sign of ESA-induced PRCA is severe resistance to treatment, which manifests as a rapid decline in hemoglobin levels to 5–6 g/dl or transfusion dependence. On exclusion of other causes of ESA hyporesponsiveness, measurement of blood cell counts should be performed; a reticulocyte count of $< 10 \times 10^9$/l in

the presence of normal white cell and platelet counts justifies bone marrow examination and measurement of anti-EPO antibodies.[3] Although bone marrow examination in a patient with ESA-induced PRCA will usually demonstrate an absence of erythroblasts, the diagnosis is confirmed by the presence of neutralizing anti-EPO antibodies.[3] The presence of circulating anti-EPO antibodies is usually determined with an immunoassay (radioimmunoprecipitation or enzyme-linked immunosorbent assay) or surface plasmon resonance methods;[3] samples that are positive for the presence of anti-EPO antibodies are then tested for the presence of EPO-NAbs using a cell-based bioassay.

Antibody-mediated PRCA is very rarely self-limiting and usually necessitates therapeutic intervention. The most important initial steps in its management are to stop the further administration of ESA therapy and to treat the anemia with red blood cell transfusions.

The therapeutic approach is based on the use of immunosuppressive therapies.[44] The most effective treatment, with almost complete recovery, is kidney transplantation.[44,45] Transplanted patients also receive immunosuppressive therapy as part of their anti-rejection protocol, and it is unclear whether the success of this strategy is related to this therapy or to the transplant itself. Corticosteroids and cyclosporin A, alone or in combination, seem beneficial.[45] Several small studies indicate that cyclosporin A alone induces rapid recovery and could be proposed as first-line therapy.[45,46] Based on these studies, typical starting doses for immunosuppressive therapies are 0.5–1.0 mg/kg/day for corticosteroids and 200 mg/day for cyclosporin; patients who respond to these therapies would be expected to recover (as indicated by transfusion independence and increases in reticulocytes or hemoglobin) within 3 months.

A challenge for nephrologists is whether ESA therapy can be re-started in patients who have recovered from PRCA with disappearance of NAbs. Re-challenge with an ESA can cause a relapse,[47] and may induce systemic reactions.[34] Successful re-challenge has been observed in isolated cases, although this should be carefully considered and should preferentially use intravenous administration.[48]

A study has shown that a novel synthetic peptide-based EPO receptor agonist (peginesatide) can correct anemia in chronic kidney disease patients with antibody-mediated PRCA.[4] This small clinical trial in 14 patients indicated a high success rate for peginesatide in rescuing patients with this condition. The biological rationale for the use of peginesatide in this setting is based on the fact that the amino-acid sequence is different from that of recombinant EPO, and thus it does not cross-react with anti-EPO antibodies.

The therapeutic recommendations for chronic kidney disease patients with ESA-induced PRCA would therefore be (Figure 2): (1) cessation of ESA therapy; (2) correction of anemia with blood transfusions if necessary; (3) consideration of kidney transplantation as the most effective treatment; (4) introduction of immunosuppressive therapy starting with cyclosporin A alone or in combination with corticosteroids, or corticosteroids with cyclophosphamide. In the future, as an alternative to immunosuppressive therapy, peginesatide could be a promising treatment, although at the time of writing this remains an unlicensed investigational drug.

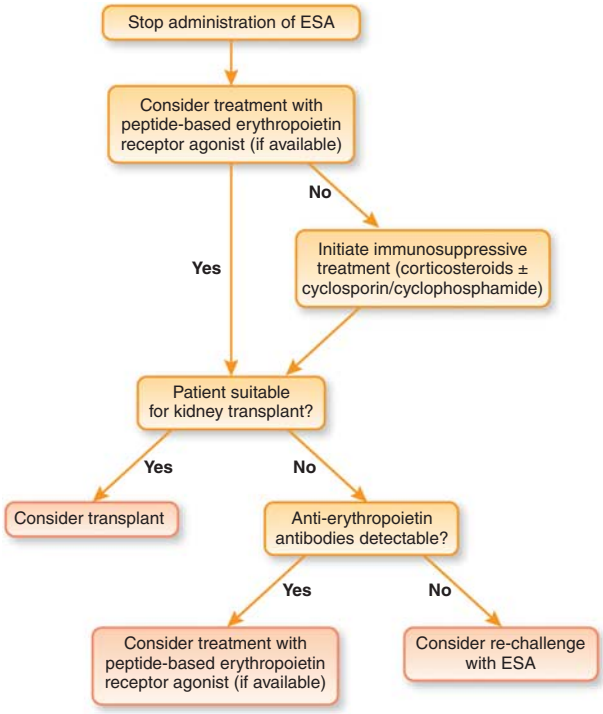

Figure 2 | Proposed treatment algorithm for erythropoiesis-stimulating agent (ESA)–induced pure red cell aplasia.

CONCLUSIONS

PRCA remains an extremely rare adverse effect of treatment with ESAs, although cases continue to emerge across the product class. As with any rare disease or complication of therapy, the pathogenesis will only be defined through close cooperation of clinicians, scientists, the pharmaceutical industry, regulators, and patients. Advances are being made in delineating the root causes of antibody-mediated PRCA, with the most recent and plausible explanation being the leaching of tungsten (from the tungsten pins used in the manufacture of glass syringes) into the syringe contents. This explanation has widespread applicability to ESA treatment and potentially to other therapeutic proteins.

Therapy to date has relied on systemic immunosuppression to impair immunological responsiveness. The use of peptide mimetics is a promising development, but of itself will not modify production of antibodies to EPO.

DISCLOSURE

All authors have variously received research grants and/or honoraria from Amgen, J&J/Ortho Biotech, Roche, Sandoz, Takeda/Affymax, and Vifor Pharma.

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