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TECHNICAL INVESTIGATIONS INTO THE CAUSE OF THE INCREASED INCIDENCE OF ANTIBODY-MEDIATED PURE RED CELL APLASIA ASSOCIATED WITH EPREX®

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The cause of the increased incidence of antibody-mediated PRCA associated with EPREX. Leachates resulting from the action of polysorbate 80 on uncoated rubber syringe stoppers were the most likely product specific cause of the increased incidence of antibody-mediated PRCA from 1998 through 2003.

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Key words:

epoetin, EPREX®, immunogenicity, pure red cell aplasia, PRCA, leachates, polysorbate 80

Abstract:

The incidence of antibody-mediated pure red cell aplasia (PRCA) in patients with chronic kidney disease began to increase in 1998 and reached a peak in 2002. Most cases were associated with EPREX® use. All of the cases involved subcutaneous administration. Risk mitigation strategies have successfully reduced the incidence of antibody-mediated PRCA. A rigorous technical investigation identified leachates extracted by polysorbate 80 from uncoated rubber syringe stoppers as the most likely cause of increased immunogenicity associated with EPREX®. This conclusion is based upon five criteria: (1) the temporal correlation between the occurrence of leachates in the product and the increased incidence of PRCA; (2) the exclusive presence of leachates in polysorbate 80 formulated EPREX® in syringes with uncoated rubber stoppers, and the absence of these leachates in other epoetin products with lower rates of PRCA; (3) the presence of leachates in microgram quantities which are sufficient to initiate rare immune responses; (4) the requirement of an adjuvant in the immune mechanism for generating a T cell mediated B cell response leading to IgG antibodies of the type detected in PRCA patients; and (5) the demonstration of the ability of the leachates to act as an adjuvant that is capable of stimulating the production of IgG antibodies in animal models.

INTRODUCTION

From 1998 onwards, the reported incidence of pure red cell aplasia (PRCA) associated with epoetin use in patients with chronic kidney disease (CKD)

increased sharply (1), from only sporadic reports over the previous 10 years (2-4) to a peak of 71 cases in 2002. Of the 262 cases of suspected epoetin-associated PRCA that have now been reported, 217

had anti-erythropoietin antibodies; 201 of those cases were associated with subcutaneous use of EPREX®. Risk mitigation strategies that include improved cold-chain management (5) and a recommen-

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dation to switch to intravenous EPREX[®] administration (followed by a formal contraindication in Europe) have significantly reduced the incidence of antibody-mediated PRCA.

Immune responses to many therapeutically used exogenous proteins have been described, and although human derived and recombinant human proteins are less immunogenic than animal proteins, immune responses to these proteins have also been described (6,7). It was unclear why the immunogenic response to erythropoietin in patients treated with EPREX[®] had apparently increased so dramatically in 1998 and this increased incidence of PRCA led to an intensified investigation of the manufacturing process and of the purity of both purified bulk and finished product. The technical investigation involved a detailed review of manufacturing data and the development and use of more sensitive analytical methods. The goal was to analyse the epoetin alfa molecule for signs of degradation or alteration and to identify any impurities that might increase the immunogenicity of the product. No irregularities in the manufacturing process or in the epoetin alfa molecule in the bulk or the finished product were identified. However, additional, previously undetected compounds were observed in an experimental high performance liquid chromatography (HPLC) elution profile of EPREX[®]. Further investigation indicated that these additional compounds were organics, termed leachates in this paper, which had been leached from the uncoated rubber syringe stopper by the action of polysorbate 80 within the EPREX[®] formulation.

This paper reports the results of the technical investigations and the evidence that strongly suggests these leachates were the product-specific cause of the increased frequency of antibody-mediated PRCA. As of April 2003, all EPREX[®] prefilled syringes with the polysorbate 80 based formulation have been shipped with

Flurotec stoppers that do not release any leachates.

METHODS

Reverse phase HPLC of EPREX[®]

Reverse phase HPLC analysis of epoetin alfa (EPREX[®]/ERYPO[®], Ortho Biotech, a division of Janssen-Cilag) syringes was carried out using a Vydac C4 column (4.6 x 250 mm) with monitoring at 214 and 280 nm. Mobile phase A consisted of 0.06% trifluoroacetic acid (TFA) in water, and mobile phase B consisted of 0.06% TFA in acetonitrile. A flow rate of 1.0 ml/minute was used for all sample analyses. Initially, after sample injection, a 10-minute hold at 0% mobile phase B was performed followed by a linear gradient of 0% to 55% of mobile phase B for 115 minutes. A 10-minute gradient to 75% mobile phase B, followed by another 10-minute gradient to 100% mobile phase B was performed. For better separation of the peaks, this method was optimised as follows. After sample injection, a 5-minute hold at 5% mobile phase B was performed followed by a linear gradient of 5% to 90% of mobile phase B for 90 minutes. Monitoring was performed at 214 and 280 nm. Concentrations of leachate peaks were estimated using bisphenol A as an internal reference standard. The samples tested consisted of EPREX[®] solutions formulated with human serum albumin (HSA) or polysorbate 80 and delivered from prefilled syringes or vials with uncoated or fluoro-resin coated stoppers (Flurotec[®], Daikyo Seiko, Ltd.), and commercially available formulations of epoetin beta (Neorecormon[®], Roche) and darbepoetin alfa (Aranesp[®], Amgen) in prefilled syringes with coated rubber stoppers.

Immunogenicity studies in mice

For ovalbumin (OVA), 6–8-week-old BDF-1 mice (Charles River Laboratories, Inc.) were injected subcutaneously on day 0 and day 14 with approximately 100 µg of OVA with the addition of leachates at concentrations ranging from 0.3 to 2.7 µg/dose. Bleeds were taken prior to the first immunization and at days 14, 21, and

28. For epoetin alfa, mice were injected weekly for 4 weeks starting on day 0 with 10 µg epoetin alfa with the addition of leachates as described above. Bleeds were taken prior to the first immunization and at days 35 and 56. Each antigen was injected alone and in combination with Incomplete Freund's Adjuvant (IFA) as controls. Serum samples were stored at –20°C until analysed by enzyme linked immunosorbent assay (ELISA).

ELISA for detection of antibodies against epoetin alfa and OVA

ELISA was performed as previously described by Braun et al. and modified for OVA (8). Briefly, 96-well plates (Nunc MaxiSorp[™]) were coated with 0.5 µg/well chicken egg OVA (Sigma Grade III, Sigma-Aldrich, Inc.) or epoetin alfa in a bicarbonate-coating buffer overnight at 4°C. Non-specific binding was blocked by incubating the plates with a phosphate buffered saline containing 1% bovine serum albumin. Following washing, dilutions of mouse serum containing antibodies (starting at 1:100) were added in duplicate and incubated with agitation for 1 hour at room temperature. The plates were washed to remove unbound antibodies and bound antibody was detected using a goat anti-mouse IgG horseradish peroxidase conjugate (Zymed Laboratories, Inc.) followed by 3,3', 5,5' tetramethylbenzidine substrate. Optical density (OD) was read at 450 nm. For OVA, mouse anti-OVA (Sigma-Aldrich, Inc.) was included as a positive control on all plates. For epoetin alfa, a standard curve of mouse anti-erythropoietin IgG was run as a positive control in all assays. Time 0 (pre-bleeds) were used as a negative control. A positive response was defined as an OD value that was at least twice the mean of the day 0 serum samples for all mice plus 3 standard deviations of the mean OD of all day 0 animals.

Hematocrit

Hematocrits were determined in these mice to establish if there was a physiological response to the adjuvants. BDF-1 mice

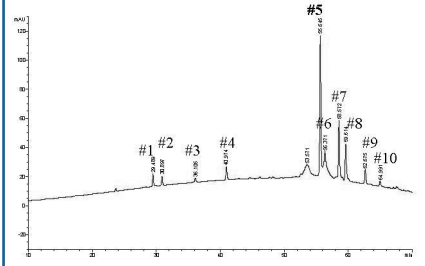
were treated as described above, except they received 40 µg epoetin alfa per dose. Hematocrits were determined prior to immunization and at days 35 and 56 by centrifugation of microcapillary hematocrit tubes in a Biofuge B. The percent packed cell volume was determined on an Adams microhematocrit reader.

RESULTS

Leachates from rubber stoppers

Several late-eluting, non-peptide peaks were observed using an experimental reverse phase HPLC in solutions delivered from prefilled syringes containing the polysorbate 80 formulation and fitted with uncoated rubber stoppers (Fig. 1). These peaks were present both in the presence of active product as well as in placebo syringes that contained no product, indicating that they were not protein related. These peaks were further resolved into 10 peaks by an optimised reverse phase HPLC method (Fig. 2). The identities of peaks 2 through 9 (shown in Table 1) were determined using mass spectrometry analyses and comparison to reverse phase HPLC

Figure 2. Optimised reverse phase HPLC profile of the placebo syringe. The leachates were resolved into 10 components; a cut-off of > 20 ppb was used. The low broad peaks to the left of peak #5 and under peak #6 are due to polysorbate 80. Absorbance was at 280 nm.

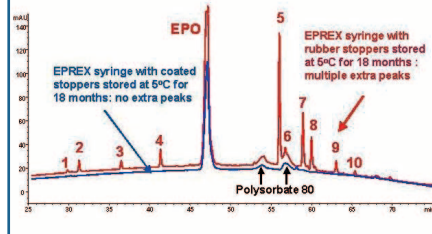


profiles of authentic standards (data not shown). Peak 1 was present in low amounts and has not been identified. All of the identified peaks contained phenolic rings that absorb at 280 nm. Peaks 3 through 10 are all potential forms or derivatives of Vultac[®], a low molecular weight multisulfide alkyl phenol that is used as a curing agent in making the rubber stoppers. Since all of the materials contained phenolic rings, the concentrations of the individual peaks were estimated by assuming that the response factors at 280 nm for each component were equivalent to that of bisphenol A that was used as a reference standard. The mean concentration of the individual components ranged from 0.024 µg/ml to 0.78 µg/ml, and the total was estimated to be from 1 to 2 µg/dose for syrin-

ges between the age of 1 to 3 years as shown in Table 1. The level of leachates increased with the storage time of the pre-filled syringes with uncoated rubber stoppers, starting at low levels and increasing to microgram per dose levels at 18–24 months.

The leachates were only found in the polysorbate 80 formulations of EPREX[®] or EPREX[®] placebo in prefilled syringes with uncoated rubber stoppers. Analyses

Figure 3. Optimised reverse phase HPLC profile comparing 18-month-old polysorbate 80 formulated EPREX[®] syringes with the uncoated rubber stopper (top curve) showing epoetin alfa (EPO) plus the 10 leachate peaks, and the Flurotec stopper (lower curve), which only shows epoetin alfa and 2 broad polysorbate 80 peaks. Absorbance was at 280 nm.



of other EPREX[®] preparations, including the EPREX[®] HSA formulation with uncoated syringe stoppers, the polysorbate 80 formulation with Flurotec syringe stoppers, vials with coated stoppers, and other

Figure 1. Reverse phase HPLC profile of A) EPREX[®] from prefilled syringes with coated rubber stoppers and the polysorbate 80 formulation; B) EPREX[®] from prefilled syringes with uncoated rubber stoppers and the polysorbate 80 formulation; and C) placebo from prefilled syringes with uncoated rubber stoppers and the polysorbate 80 formulation. The large peak at approximately 105 minutes is epoetin alfa. The presence of extra peaks in the chromatogram is only observed with the uncoated rubber stoppers. Absorbance was at 214 nm.

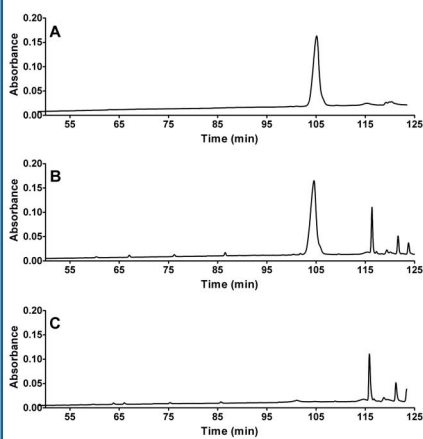


Table 1. Identification and average concentrations of the purified leachates 1 through 10 (from reverse phase HPLC) based on bisphenol A used as an internal standard. An average of 6 representative lots aging from 1 to 3 years was used for calculating concentration of the leachates

Peak ^a	Compound	Average concentration ^b
1	Unknown	Unknown
2	Bisphenol A	0.070
3	4- <i>tert</i> -amylphenol	0.046
4	2-chloro-4- <i>tert</i> -amylphenol	0.037
5	Vultac [®] 2 disulfide	0.778
6	2,2'-methylene-bis-4- <i>tert</i> -amylphenol	0.243
7	Vultac [®] 2 trisulfide	0.235
8	Vultac [®] 2 tetrasulfide	0.142
9	Vultac [®] 2 pentasulfide	0.063
10	Vultac [®] 2 hexasulfide	0.024

a From Figure 2

b Units are µg/mL = ppm

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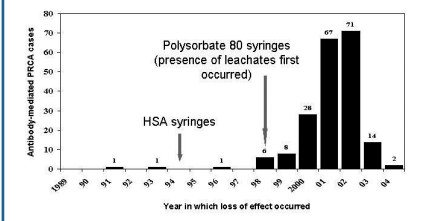
epoetin products such as epoetin beta and darbepoetin alfa with coated syringe stoppers did not show these leachate peaks (Table 2). None of the other products tested were associated with the increased frequency of PRCA; the presence of leachates was a unique factor associated with PRCA. Figure 3 shows HPLC chromatograms comparing 18-month-old syringes of EPREX[®] with the uncoated rubber stopper to the Flurotec stopper. As can be seen, there are no prominent leachate peaks with the Flurotec stoppers.

Correlation of leachates with increased frequency of PRCA

Figure 4 shows the number of cases of PRCA associated with EPREX[®] by year. The EPREX[®] prefilled syringes were originally launched in 1994 with an HSA-based formulation with uncoated rubber stoppers. Leachates have not been detected with these syringes, and the frequency of PRCA was similar to the low rate seen with all epoetin products. In 1998, the formulation was revised, with polysorbate 80 replacing HSA as the stabilizer in the same syringes with uncoated stoppers. This is the formulation–syringe combination in which leachates occur and which correlates with the increase in the frequency of PRCA.

To limit the increasing incidence of antibody-mediated PRCA, the manufacturer, in collaboration with the health authorities, initiated three risk mitigation strategies: (1) instituting tighter control of the product's cold-chain distribution; (2) recommending a switch to the lower risk route of intravenous administration in CKD patients, including a formal contraindication of subcutaneous administration by health authorities in Europe in December of 2002; and (3) the conversion, which occurred in April 2003, of all prefilled syringes with the polysorbate 80 formulated product to Flurotec stoppers. As shown in Figure 4, the frequency of PRCA has dropped significantly and has returned to a baseline level for 2004, indicating the success of these mitigation strategies.

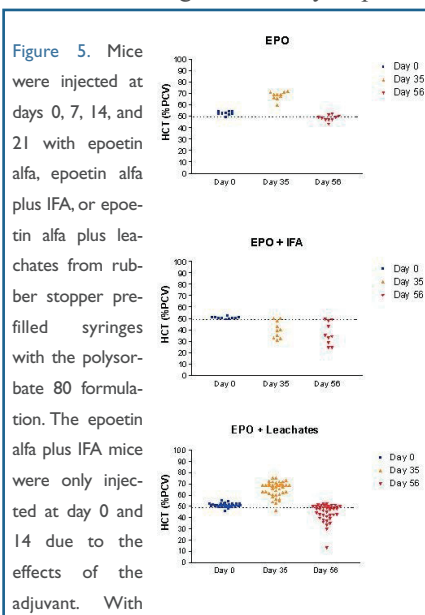
Figure 4. Number of antibody positive PRCA cases by year. Note that there was a very low baseline level between 1989 and 1997 with no increase with the introduction of prefilled syringes with the HSA formulation in 1994. The polysorbate 80 formulated prefilled syringes were introduced in 1998 and correlate with the increase in the number of cases of antibody positive PRCA. It has subsequently been demonstrated that leachates accumulate in these prefilled syringes with uncoated syringe stoppers over the shelf life of the product.



Immune response in mice

Mouse studies were performed to determine if leachates could act as adjuvants and stimulate an increased immune response. Studies were conducted with epoetin alfa as the antigen. Antibody responses

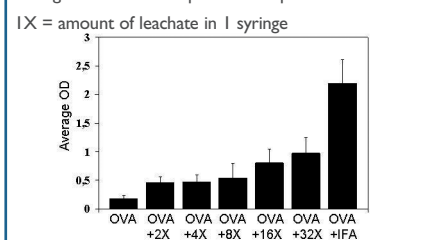
Figure 5. Mice were injected at days 0, 7, 14, and 21 with epoetin alfa, epoetin alfa plus IFA, or epoetin alfa plus leachates from rubber stopper prefilled syringes with the polysorbate 80 formulation. The epoetin alfa plus IFA mice were only injected at day 0 and 14 due to the effects of the adjuvant. With epoetin alfa alone, the mice exhibited an increase in hematocrit due to the additional epoetin alfa (40 µg/dose) that returned to normal by day 56. Mice administered epoetin alfa plus IFA showed a decline in hematocrit that became more severe by day 56. Mice administered epoetin alfa plus leachates exhibited a more variable increase to no increase in hematocrit at day 35 and returned to normal or subnormal levels by day 56.



in this model proved very complex to interpret. The high doses of epoetin alfa administered as the antigen (4 weekly doses of 40 µg/dose) significantly increased the hematocrit at day 35 (Fig. 5). The higher hematocrits returned to normal by day 56. Mice injected with epoetin alfa plus IFA, a strong adjuvant, demonstrated no increase in hematocrit and in many cases showed a decrease at day 35 that became more severe by day 56. This is likely due to the generation of neutralizing antibodies against epoetin alfa that cross-react with mouse erythropoietin causing a PRCA like condition. IgG antibodies against epoetin alfa were detected in most mice treated with epoetin alfa plus IFA. In the presence of leachates, which are a weak adjuvant, the mice demonstrated a rise in hematocrit with some mice showing less of a rise than the epoetin alfa control at day 35. By day 56, the hematocrit for these mice had returned to normal with some mice showing a decreased hematocrit similar to that seen in animals treated with epoetin alfa plus IFA.

The hematocrit data support the idea that the leachates are acting as an adjuvant in

Figure 6. Adjuvant activity of leachates. Ovalbumin (OVA), OVA plus leachates, or OVA plus IFA were injected into BDF-1 mice. The mice were bled on days 0, 14, 21, and 28. The results of the ELISA assay for day 28 serum samples are shown. The data are expressed as the average OD of the positive responses ± 2 SEM.



this model. In the case of epoetin alfa plus IFA, antibodies against epoetin alfa could be detected. However, antibodies against epoetin alfa were not readily detected with the leachates, perhaps due to a combination of a much weaker antibody response and interference from the mouse erythropoietin in the antibody assay.

Studies conducted using OVA as an antigen confirmed that the leachates could stimulate a stronger response to the OVA in mice than was seen with OVA alone, and a dose-dependent adjuvant effect of the leachates was observed (Fig. 6), demonstrating the potential of the leachates to act as adjuvants and stimulate an increased immune response.

It is well documented that the initiation of a T cell mediated B cell reaction to generate the IgG type of antibodies against epoetin alfa that are seen in PRCA patients (9) requires the presence of an adjuvant (10). The potential for leachates from rubber or plastic materials to act as immune stimulators is well known in the literature. (11). The ability of polysorbates and other non-ionic detergents to leach materials out of plastics and rubber materials has also been documented (12). In addition, sensitisation to leachates from synthetic rubber tubing used in medical equipment (13) may predispose patients to hypersensitivity reactions during subsequent contact with synthetic rubber products. Hemodialysis patients are particularly likely to be exposed to such sensitisers (14), and may be more likely to mount an immune response to epoetin in the presence of synthetic rubber leachates. A product specific adjuvant like the leachates detected in EPREX® could be sufficient to generate the increased frequency of immunogenicity as discussed above.

DISCUSSION

Any hypothesis concerning the cause of antibody-mediated PRCA must take into account the timing of the increase in incidence of PRCA. The only change in formulation, structure, or purity that had a temporal relationship with the increased incidence was the replacement of HSA with polysorbate 80 in 1998. Rubber stoppers were in use from the first introduction of EPREX® syringes for subcutaneous administration in 1994. The replacement of HSA with the non-ionic detergent polysorbate 80 caused a change by leaching of organic materials from the uncoated rubber

stoppers. The prefilled syringes that were on the market that contained this combination were the 1,000, 2,000, 3,000, 4,000, and 10,000 unit presentations. Although epoetin beta and darbepoetin alfa both contain polysorbate stabilisers, syringes for subcutaneous injection of these products have coated stoppers. EPREX® prefilled syringe presentations containing 5,000, 6,000, 7,000, 8,000, and 9,000 units were launched in 2001 with Flurotec stoppers. The 10 leachate peaks were not detected in any product with coated stoppers, despite the presence of polysorbate stabilisers.

Mouse studies were performed to examine the role of leachates in relation to immunogenicity. In early studies conducted using the leachates with epoetin alfa some mice exhibited a PRCA like effect, characterized by a decrease in hematocrit. However the authors had concerns about testing with epoetin alfa as the immunogen as it creates a complex set of physiological responses in the mice that appeared to interfere with the measurement of antibodies against epoetin. Therefore, the well characterized surrogate antigen OVA was used to test the adjuvant effect of the leachates from prefilled syringes of

EPREX® containing uncoated rubber syringe stoppers. A dose-ranging study conducted with leachates isolated from prefilled syringes of EPREX® and using OVA as the antigen clearly demonstrated the adjuvant effect of the leachates.

Extensive studies were conducted to look for changes in the epoetin alfa molecule in both the bulk and finished product. Specifically, methods were employed to investigate aggregation, oxidation, deamidation, carbohydrate composition, denaturation, and sequence/structural changes. None of these analyses demonstrated any change in the product that was outside of historical experience prior to the increase in PRCA.

Studies were conducted to investigate whether the silicone oil that is used to lubricate all prefilled syringes could act as an adjuvant. No positive adjuvant response was detected in mice in studies looking at increasing concentrations of silicone. The silicone process also has not changed since the syringes were introduced in 1994 and is identical for current HSA containing syringes, which show a low baseline rate of PRCA, as well as for the Flurotec stopper syringes.

Table 2. Relationship of the presence of leachates to the type of stabilizer and stopper in epoetin formulations

Epoetin preparation	Type of stopper	Polysorbate 80 in formulation	Presence of leachates
EPREX® syringes			
EPREX® placebo	Uncoated	Yes	Yes
1000, 2000, 3000, 4000, 10,000 ^a IU	Uncoated	Yes	Yes
5000–9000 IU	Coated	Yes	No
HSA	Uncoated	No	No
EPREX® vials			
10,000 IU	Coated	Yes	No
40,000 IU	Coated	Yes	No
Comparator products			
Epoetin alfa USA	Coated	No	No
Epoetin beta	Coated	Yes ^b	No
Darbepoetin alfa	Coated	Yes	No

^a Pre-April 2003 syringe stoppers were uncoated; Post-April 2003 syringe stoppers are coated.

^b Polysorbate 20, all other 'Yes' entries indicate polysorbate 80.

Studies were also conducted to look for a possible adjuvant effect that might occur due to the polysorbate 80 in the EPREX[®] formulation. Different levels of polysorbate 80, including significantly higher levels than the EPREX[®] formulation, did not demonstrate an increase in immunogenicity in mice. Early clinical data indicate that the increase in PRCA was not associated with the combination of polysorbate 80 formulation with the Flurotec stopper, indicating that polysorbate 80 alone is not an adjuvant. This is in agreement with Vogel et al., which reports, “polysorbate 80 has no adjuvant properties on its own” (15).

It was recently hypothesized that EPREX[®] might associate with micelles of polysorbate 80 in the EPREX[®] formulation (16). It was implied that association between polysorbate 80 micelles and epoetin alfa could be a cause of PRCA. Investigations by the authors using light scattering clearly indicated that epoetin alfa does not form macromolecular structures with micelles of polysorbate 80. It was also

demonstrated that polysorbate 80 micelles are extremely unstable, dissipating within seconds upon dilution (17). Kerwin et al. also investigated whether an interaction between polysorbate 80 or polysorbate 20 micelles and epoetin could form macrostructures or aggregates and reported at the meeting of the European Renal Association – European Dialysis and Transplant Association in May, 2004, that “based on analytical ultracentrifugation data, neither polysorbate 20 or polysorbate 80 form micellar-like aggregates with darbepoetin alfa or epoetin alfa” (18).

CONCLUSION

This technical investigation indicates that leachates resulting from the action of polysorbate 80 on uncoated rubber syringe stoppers are the most likely product-specific cause of the increased frequency of PRCA that occurred with EPREX[®] between 1998 and 2003. A thorough investigation of the epoetin molecule in both the bulk and the finished products demonstrated a high degree of consistency. No chan-

ges were detected in the epoetin alfa molecule that were outside of the normal ranges over the history of the product or that were different from other epoetin products that demonstrated only a baseline frequency of PRCA. Investigation of the stabilizer polysorbate 80 failed to demonstrate any evidence of an adjuvant effect, confirming the known safety profile of the polysorbate 80 as an excipient for protein formulations. Silicone oil also failed to show any adjuvant effect in mice and could be excluded based on the lack of any temporal association between silicone oil and PRCA. Several other hypotheses were investigated over the course of the investigation. Leachates were shown to have a dose-dependent adjuvant effect in a mouse model. Based on the evidence, only the presence of leachates, which occurred between 1998 and 2003 in the polysorbate prefilled syringes with the uncoated rubber stoppers, fit all of the criteria as the causative factor for the increased incidence of antibody-mediated PRCA.

REFERENCES

- Casadevall N, Nataf J, Viron B et al. Pure red-cell aplasia and antierythropoietin antibodies in patients treated with recombinant erythropoietin. *New England J Med* 2002; 346(7): 469-475.
- Bergrem H, Danielson BG, Eckardt KU, Kurtz A, Stridsberg M. A case of antierythropoietin antibodies following recombinant human erythropoietin treatment. *Molecular Physiol Clin Appl* 1993; 265: 273.
- Peces R, Torre MdI, Alcázar R, Urria JM. Antibodies against recombinant human erythropoietin in a patient with erythropoietin-resistant anemia. *New England J Med* 1996; 335(7): 523-524.
- Prabhakar SS, Muhlfelder T. Antibodies to recombinant human erythropoietin causing pure red cell aplasia. *Clin Nephrol* 1997; 47(5): 331-335.
- Wegewijs HW. The supply chain for biopharmaceuticals: a manufacturer's view. *Eur J Hosp Pharm* 2003; 1: 86-88.
- Chamberlain P. Immunogenicity of therapeutic proteins. Part 1. Causes and clinical manifestations of immunogenicity. *Reg Rev* 2002; 5(5): 4-9.
- Koren E, Zuckerman LA, Mire-Sluis AR. Immune responses to therapeutic proteins in humans—clinical significance, assessment and prediction. *Curr Pharm Biotechnol* 2002; 3(4): 349-360.
- Braun A, Kwee L, Labow MA, Alsenz J. Protein aggregates seem to play a key role among the parameters influencing the antigenicity of interferon alpha (IFN-alpha) in normal and transgenic mice. *Pharm Res* 1997; 14(10): 1472-1478.
- Casadevall N, Nataf J, Viron B et al. Pure red-cell aplasia and antierythropoietin antibodies in patients treated with recombinant erythropoietin. *New England J Med* 2002; 346(7): 469-475.
- Abbas AK, Lichtman AH. *Cellular and Molecular Immunology*. Philadelphia: WB Saunders, 2003.
- Primeau MN, Adkinson NF, Jr., Hamilton RG. Natural rubber pharmaceutical vial closures release latex allergens that produce skin reactions. *J Allergy Clin Immunol* 2001; 107(6): 958-962.
- Jenke D. Extractable/leachable substances from plastic materials used as pharmaceutical product containers/devices. *PDA J Pharm Sci Technol* 2002; 56(6): 332-371.
- Hill SS, Shaw BR, Wu AHB. Plasticizers, antioxidants, and other contaminants found in air delivered by PVC tubing used in respiratory therapy. *Biomed Chromatography* 2003; 17(4): 250-262.
- Hill SS, Shaw BR, Wu AH. The clinical effects of plasticizers, antioxidants, and other contaminants in medical polyvinylchloride tubing during respiratory and non-respiratory exposure. *Clin Chim Acta* 2001; 304(1-2): 1-8.
- Vogel FR, Powell M, Alving C. *Compendium of Vaccine Adjuvants and Excipients*. <http://www.niaid.nih.gov/daids/vaccine/pdf/compendium.pdf> (Accessed 28 July, 2004).
- Hermeling S, Schellekens H, Crommelin DJA, Jiskoot W. Micelle-associated protein in epoetin formulations: a risk factor for immunogenicity? *Pharm Res* 2003; 20(12): 1903-1907.
- Heavner GA, Villalobos S, Gunturi R, Brennan P. Interaction of Polysorbate 80 with Erythropoietin. 18th Annual Symposium of the Protein Society, San Diego, CA, August, 2004.
- Kerwin B, Deechongkit S, Park S, Kim J, Burnett H. Effects of polysorbates 20 and 80 on the structure and stability of darbepoetin alfa and epoetin alfa. *European Renal Association-European Dialysis and Transplant Association (ERA-EDTA)*, 17 May 2004: 321.