Micelle-Associated Protein in Epoetin Formulations: A Risk Factor for Immunogenicity?

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Purpose. An upsurge of pure red cell aplasia (PRCA) cases associated with subcutaneous treatment with epoetin alpha has been reported. A formulation change introduced in 1998 is suspected to be the reason for the induction of antibodies that also neutralize the native protein. The aim of this study was to detect the mechanism by which the new formulation may induce these antibodies.
Methods. Formulations of epoetin were subjected to gel permeation chromatography with UV detection, and the fractions were analyzed by an immunoassay for the presence of epoetin.
Results. The chromatograms showed that Eprex®/Erypo® contained micelles of Tween 80. A minute amount of epoetin (0.008–0.033% of the total epoetin content) coeluted with the micelles, as evidenced by ELISA. When 0.03% (w/v) Tween 80, corresponding to the concentration in the formulation, was added to the elution medium, the percentage of epoetin eluting before the main peak was 0.68%.
Conclusions. Eprex®/Erypo® contains micelle-associated epoetin, which may be a risk factor for the development of antibodies against epoetin.

KEY WORDS: epoetin, erythropoietin, immunogenicity, micelles, protein formulation, Tween

INTRODUCTION

Over 80 recombinant human proteins are currently on the European market (1), several of which have been shown to induce antibody formation in patients (2,3). There are many factors that contribute to the immunogenicity of therapeutic proteins, such as the route of administration, patient characteristics, dose, and duration of therapy. Moreover, the formulation and the structure of the protein may influence the immune response (2,4). For instance, therapeutic protein formulations containing protein aggregates are more immunogenic than formulations without aggregates (5–8).

Immunogenicity of recombinant human proteins can have serious clinical consequences. A good example is the antibody formation reported during treatment with recombinant human erythropoietin (epoetin). The formed antibodies not only block the therapeutic effect but also neutralize the endogenous protein, resulting in pure red cell aplasia (PRCA) (9–12). The patients become transfusion dependent, some for prolonged periods of time. The majority of the reported PRCA cases occurred after 1998 in European patients who had received epoetin alpha subcutaneously in a new formulation. Only a few cases were reported to be associated with epoetin beta.

Endogenous erythropoietin and epoetin are heavily glycosylated: the molecular mass of the peptidic part is 18.2 kDa; the sugars increase the molecular mass to 30.4 kDa. Erythropoietin, epoetin alpha, and epoetin beta differ slightly in their glycosylation patterns. However, it has been shown that the antibodies formed in the patients are not directed against the sugar chains of the protein (10,12). Therefore, glycosylation differences are not expected to play a key role in the observed immunogenicity problem. Instead, differences in the formulation are more likely to influence the immunogenicity of epoetin. Table I illustrates that substantial differences exist in the composition of epoetin alpha (Erypo®, Eprex®) and epoetin beta (NeoRecormon®) formulations.

In this study we analyzed formulations of epoetin alpha and beta for potential interactions between the protein and excipients. It is shown that the epoetin alpha formulation contains small quantities of micelle-associated epoetin. This micellar form of epoetin may be an important risk factor for the development of antibodies in patients.

MATERIALS AND METHODS

Epoetin Formulations

Erypo® (Ortho Biotech, Neuss, Germany), Eprex® (Ortho Biotech, Tilburg, The Netherlands) and NeoRecormon® (Roche, Mannheim, Germany; Mijdrecht, The Netherlands) were used throughout the study. For all experiments ready-to-use syringes were used (see Table II). Eprex® and Erypo® are produced by Ortho Biotech; they contain the same excipients (see Table I) and were considered to be equivalent.

Gel Permeation Chromatography

A Superdex 200 HR 10/30 column (Amersham, Roosendaal, The Netherlands) was used for gel permeation chromatography (GPC) analyses. The mobile phase consisted of 50 mM sodium phosphate buffer, pH 6.8, and 300 mM NaCl (unless stated otherwise) and was passed through a 0.45-μm filter before use. The mobile phase was delivered to the column at a flow rate of 0.50 ml/min by a Waters 600 controller equipped with an autosampler (model 717, Waters). Chromatograms were recorded with a photodiode array detector (model 996, Waters). The column was calibrated by analyzing protein standards obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands) with known molecular weights (i.e., thyroglobulin, bovine serum albumin, ovalbumin, α-chymotrypsin, and myoglobin).

Samples of Erypo®, Eprex®, and NeoRecormon® (1500 IU/sample) were applied on the GPC column without dilution or any other pretreatment. In addition, Eprex® spiked with 0.03% (w/v) Tween 80 (Serva, Heidelberg, Germany), NeoRecormon® spiked with 0.01% (w/v) Tween 20 (Roche, Mannheim, Germany), and aqueous solutions of Tween 80 and Tween 20 were analyzed. In a separate experiment, Eprex® and NeoRecormon® were analyzed by GPC after addition of

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ABBREVIATIONS: CMC, critical micelle concentration; epoetin, recombinant human erythropoietin; GPC, gel permeation chromatography; PRCA, pure red cell aplasia.

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Table I. Qualitative Composition of Eprex®/Erypo® and NeoRecormon®

Eprex®/Erypo®	NeoRecormon®
Epoetin alpha	Epoetin beta
Glycine	Urea
Tween 80	Tween 20
Sodium chloride	Sodium chloride
Sodium phosphate	Sodium phosphate
Sodium citrate	Calcium chloride
	Glycine
	Leucine
	Isoleucine
	Threonine
	Glutamic acid
	Phenylalanine

0.03% (w/v) Tween 80 or 0.01% (w/v) Tween 20, respectively, to the mobile phase.

Fractions (0.25 ml) were collected from 10 until 35 min after injection and analyzed for the presence of epoetin by an immunoassay (see below).

Immunoassay

Human erythropoietin-specific immunoassays obtained from Roche (Penzberg, Germany) or Quantikine IVD (R&D Systems, Abingdon, UK) were used for analysis of the GPC fractions, according to the manufacturer's recommendations.

Quantikine IVD Immunoassay

One hundred microliters of assay diluent (supplied by the manufacturer) was added to the wells of a microplate coated with a murine monoclonal antibody against epoetin. Subsequently, 80 μl of dilution buffer (supplied by the manufacturer) and 20 μl of each fraction was added to a well. The plate was incubated for 1 h at room temperature under constant orbital shaking. Contents were discarded, and the wells were carefully tapped dry on a tissue. A solution of rabbit polyclonal antiepoetin antibodies conjugated to horseradish peroxidase was added to each well, and the plate was incubated on an orbital shaker at room temperature for 1 h. Contents were discarded, and the wells were washed four times with 400 μl of the supplied wash buffer. After the last wash the plate was tapped dry on a tissue. Two hundred microliters of substrate solution was added to each well. After incubation for 20–25 min, 100 μl of stop solution was added to all wells, and the absorbance was read on a Novapath microplate reader (Biorad) at a wavelength of 450 nm and a reference wavelength of 595 nm.

Roche Immunoassay

A biotinylated antiepoetin antibody solution (125 μl) was added to a streptavidin-coated microplate. The plate was incubated for 1 h at room temperature under constant orbital shaking. Contents were discarded, and the wells were washed three times with 300 μl of wash buffer [40 mM potassium phosphate buffer, pH 7.2; 0.1% (w/v) Tween 20]. After the last wash the wells were carefully tapped dry on a tissue, and 100 μl of sample buffer (supplied by the manufacturer) and 20 μl of each fraction was added to a well. The plate was incubated for 1 h at room temperature under constant orbital shaking. Contents were discarded, and the wells were washed three times with 300 μl of wash buffer. After the last wash the wells were carefully tapped dry on a tissue. A solution of rabbit polyclonal antiepoetin antibodies conjugated to horseradish peroxidase was added to each well, and the plate was incubated on an orbital shaker at room temperature for 2 h. Contents were discarded, and the wells were washed three times with 300 μl of wash buffer. After the last wash the plate was tapped dry on a tissue, and 100 μl of substrate solution was added to the wells; after incubation for 20 min the absorbance was read on a Novapath microplate reader (Biorad) at a wavelength of 405 nm and a reference wavelength of 490 nm.

To determine the epoetin concentration in the fractions, a calibration curve (made of the corresponding formulation analyzed by GPC) was included in the immunoassay. During all incubation steps the plate was covered with adhesive tape. Tween 80 and Tween 20 did not interfere in the analyses.

RESULTS

Three batches of Eprex®/Erypo® and three batches of NeoRecormon® were tested in duplicate. Representative GPC profiles of Eprex®/Erypo® and NeoRecormon® are shown in Fig. 1A,B. Both Eprex®/Erypo® and NeoRecormon® batches showed a main peak corresponding to free

Table II. Results of GPC-Immunoassays of Epoetin Formulations Tested

Product (batch nr.)	Addition to mobile phase	Concentration	Epoetin in peak 2[a,b]	
			1st run	2nd run
Erypo® (02DS14T)	None	4000 IU/0.4 ml	0.031%	—[c]
Eprex® (02CS05T)	None	4000 IU/0.4 ml	0.008%	0.014%
Eprex® (02GS05T)	None	4000 IU/0.4 ml	0.033%	0.033%
NeoRecormon® (MH6410602)	None	3000 IU/0.3 ml	n.d.[d]	n.d.
NeoRecormon® (MH6459408)	None	4000 IU/0.3 ml	n.d.	n.d.
NeoRecormon® (MH6602301)	None	4000 IU/0.3 ml	n.d.	n.d.
Eprex® (02CS05T)	0.03% Tween 80	4000 IU/0.4 ml	0.64%	0.72%
NeoRecormon® (MH6410602)	0.01% Tween 20	3000 IU/0.3 ml	0.051%	0.052%

[a] For the position of peak 2, see Fig. 1.
[b] Percentage of amount applied to the GPC column.
[c] —, not determined.
[d] n.d., not detectable.

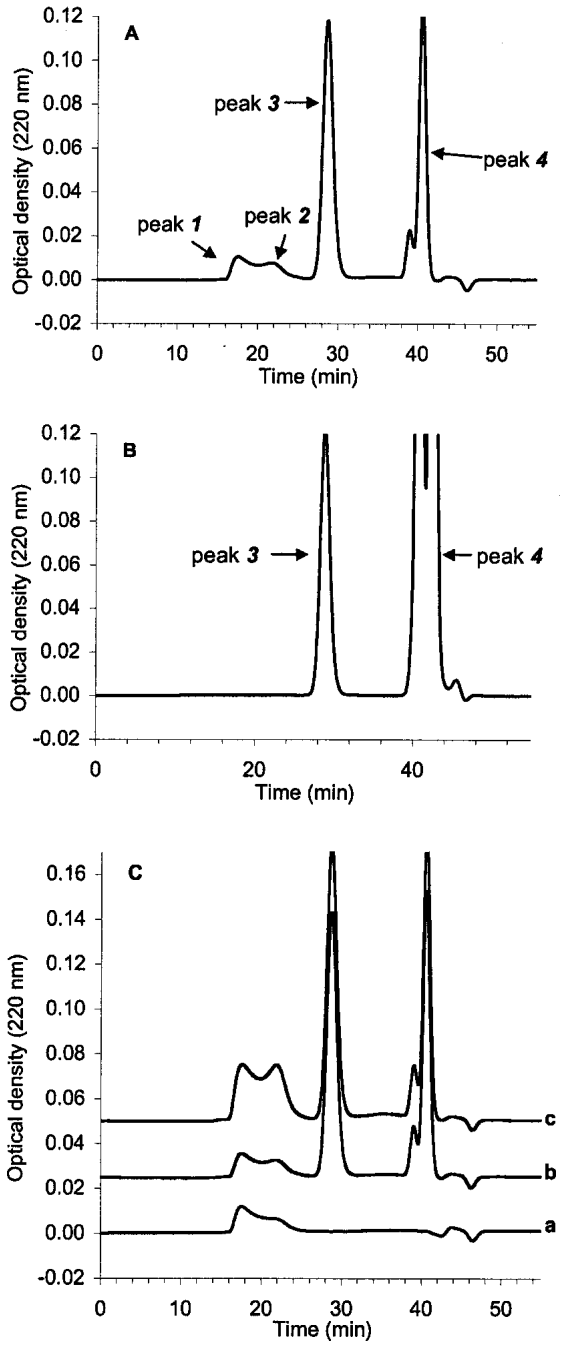

Fig. 1. GPC profiles obtained with a mobile phase consisting of 50 mM sodium phosphate buffer, pH 6.8; 300 mM NaCl. (A) Eprex®. (B) NeoRecormon®. Peak 1 has an apparent molecular weight of 670 kDa, peak 2 has an apparent molecular weight of 255 kDa and peak 3 has an apparent molecular weight of 55 kDa; peak 4 contains low-molecular-weight material. (C) Tween 80, 0.03% (w/v) (a), Eprex® (b), and Eprex® spiked with 0.03% (w/v) Tween 80 (c). Different baseline offsets are represented for sake of clarity.

epoetin (peak 3 in Fig. 1A,B). The most striking difference between the GPC profiles of Eprex®/Erypo® and NeoRecormon® is the presence of two overlapping peaks (peaks 1 and 2 in Fig. 1A,B) with a shorter retention time than the free epoetin peak (peak 3) for all Eprex®/Erypo® batches tested. These two peaks (with apparent molecular weights of 670 kDa and 255 kDa) are indicative of high-molecular-weight material present in the Eprex®/Erypo® batches. None of the NeoRecormon® batches showed peaks eluting before the free epoetin peak. Another difference between Eprex®/Erypo® and NeoRecormon® was the area of peak 4 (eluting at or near the total column volume, see Fig. 1A,B), which was much smaller for Eprex®/Erypo® than for NeoRecormon®. This is because of the larger number of low-molecular-weight excipients in the latter formulation that show UV absorbance (see Table I).

The appearance of the high-molecular-weight peaks in the Eprex®/Erypo® batches was thought to be caused by the presence of Tween 80 micelles and/or the presence of epoetin oligomers. Both possibilities were investigated. Eprex®/Erypo® contains 0.03% (w/v) Tween 80 [approximately 20 times the critical micelle concentration (CMC) (13)], and NeoRecormon® contains 0.01% (w/v) Tween 20 [approximately 1.5 times the CMC (13)]. When an aqueous solution of 0.03% (w/v) Tween 80 was injected onto the GPC column, the chromatogram showed two overlapping peaks with similar retention times and comparable peak shapes and areas as peaks 1 and 2 of Eprex®/Erypo® (see Fig. 1C). At lower concentrations of Tween 80 (below the CMC), these peaks were absent (data not shown). Moreover, Fig. 1C shows that the size of these peaks was about doubled when Eprex® was spiked with 0.03% (w/v) Tween 80. These data show that Eprex®/Erypo® indeed contains micelles of Tween 80. When NeoRecormon® was spiked with 0.01% (w/v) Tween 20, no differences between the spiked and nonspiked GPC profile could be observed. Micelles of Tween 20 could be detected only when concentrations of 0.05% (w/v) or higher were injected onto the column (data not shown).

To investigate the possible presence of epoetin in the high-molecular-weight fractions, the GPC fractions were analyzed by immunoassay. Figure 2 shows the results of one batch of Erypo® and one batch of NeoRecormon®, both of which are representative for all Eprex®/Erypo® and NeoRecormon® batches tested, respectively. Besides the main peak 3, Erypo® contained a small amount of epoetin coeluting with micellar Tween 80, i.e., at a position corresponding to peak 2 (for the position of the peaks see Fig. 1A). The amount of epoetin in peak 2 of Eprex®/Erypo® varied among batches and was calculated to be between 0.008% and 0.033% of the

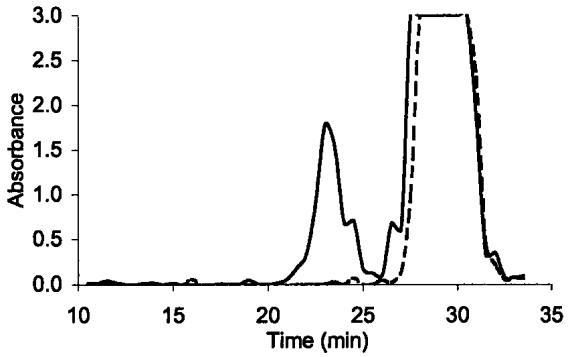

Fig. 2. Results of the immunoassay of Eprex® (*solid line*) and NeoRecormon® (*dashed line*) showing the epoetin activity in the fractions. The time reflects the time after injection. The fractions were collected during the GPC analyses with a mobile phase consisting of 50 mM sodium phosphate buffer, pH 6.8, 300 mM NaCl. The maximum absorbance of the microplate reader was 3.

total dose analyzed (see Table II). This extremely small amount hardly contributes to the optical density at 220 nm, which explains why the peak shapes and areas of the GPC profile of 0.03% (w/v) Tween 80 were virtually the same as those of peaks 1 and 2 of Eprex®/Erypo® batches (Fig. 1C). For all NeoRecormon® batches, epoetin was only detected in peak 3 (Fig. 2).

From these results it can be concluded that Eprex®/Erypo® batches contain Tween 80 micelles and a small amount of epoetin coeluting with the micelles. Neither micelles nor oligomeric epoetin was detected in NeoRecormon® batches. However, micelles are dynamic systems: surfactant monomers free in solution are in equilibrium with micellar surfactant. During the GPC procedure the (large) micelles will continuously be separated from the free (monomeric) surfactant molecules, which will shift the equilibrium between micellar surfactant and free surfactant to the monomers free in solution. If the epoetin molecules coeluting with the Tween 80 micelles were solubilized in the micelles, the amount of micellar epoetin present in the formulations would be underestimated by the above GPC procedure. Therefore, we also analyzed Eprex® and NeoRecormon® after adding 0.03% (w/v) Tween 80 and 0.01% (w/v) Tween 20 to the mobile phase, respectively. Here the micelle–monomer equilibrium during the GPC run will be kept constant, which will result in GPC/immunoassay profiles that better represent the contents of the syringes. The chromatograms of the GPC analyses in the presence of Tween are shown in Fig. 3. For Eprex® a peak eluting at a position similar to that of peak ***2*** (cf. Fig. 1A) in front of the main epoetin peak was observed. Because Tween 80 is not detected by this procedure (because the Tween 80 concentration in the sample equals that of the mobile phase), the peak probably reflects epoetin. This was confirmed by immunoassay, which showed that the amount of epoetin in these fractions (corresponding to the position of peak 2) had increased from 0.008–0.033% to approximately 0.68% (see also Table II). For NeoRecormon® a very small peak eluted in front of the main epoetin peak (see Fig. 3). This peak contained epoetin (0.052% of total amount applied to the column).

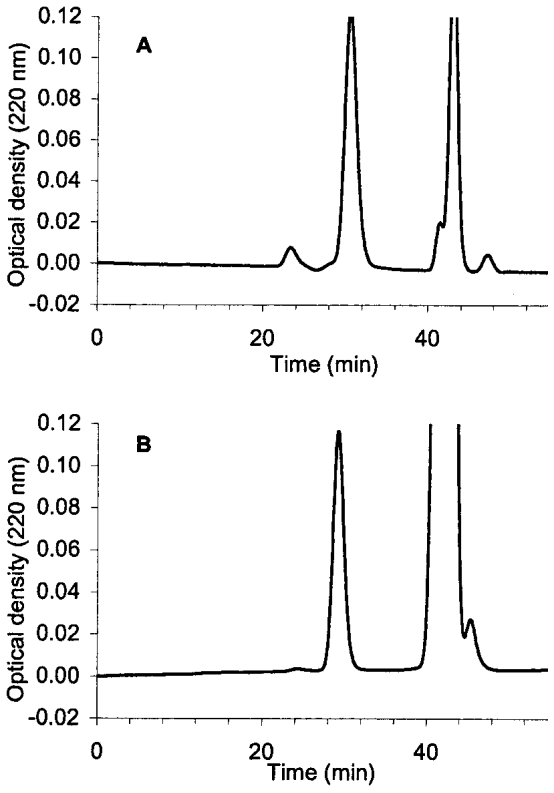

Fig. 3. GPC profile of Eprex® with a mobile phase consisting of 50 mM sodium phosphate buffer, pH 6.8, 300 mM NaCl; 0.03% (w/v) Tween 80 (A) and NeoRecormon® with a mobile phase consisting of 50 mM sodium phosphate buffer, pH 6.8, 300 mM NaCl, 0.01% (w/v) Tween 20 (B). The typical micellar peak as seen in Fig. 1 for Eprex®/Erypo® is no longer visible in panel A.

DISCUSSION

We investigated whether differences between Eprex®/Erypo® and NeoRecormon® formulations could be found that might be correlated with their immunogenicity observed in patients. The formulations of Eprex® and NeoRecormon® are substantially different (Table I). On the Swissmedic website (*http://www.swissmedic.ch*) it is reported that the estimated incidence of antibody-positive PRCA cases per 10,000 patient years for patients treated exclusively with Eprex® was 0.03 in 1989–1997 (the period before the change in the formulation of Eprex®) and 1.24 in 1998–2002. For patients treated exclusively with NeoRecormon® these values are 0.1 (1990–1997) and 0.14 (1998–2002). All cases (Eprex® and NeoRecormon®) occurred after s.c. administration. Considering the possible role of multimeric antigen presentation in breaking the immune tolerance against endogenous proteins (14), we focused on the differences in type and concentration of surfactants. Eprex®/Erypo® contains a Tween 80 concentration [0.03% (w/v)] far above its CMC, NeoRecormon® Tween 20 at a concentration [0.01% (w/v)] only slightly above its CMC.

We clearly showed that Eprex®/Erypo® contains not only monomeric epoetin but also an amount of the protein eluting in high-molecular-weight fractions on GPC. For NeoRecormon® only, after addition of Tween 20 to the eluent, minute amounts of epoetin eluted before the main peak. At least two possibilities have to be considered that might explain the presence of epoetin in the high-molecular-weight fractions: (a) epoetin oligomers coincidentally coeluting with Tween 80 micelles; (b) oligomers or monomers solubilized in or attached to Tween 80 micelles. The first option seems unlikely because the addition of Tween to the mobile phase led to an increase in the amount of epoetin in peak 2 (see Table II).

The second explanation assumes that the Tween 80 micelles contained several epoetin molecules. The average apparent molecular weight of the fractions containing micelle-associated epoetin was ca. 255 kDa. Thus, at least a few epoetin molecules can be present in one micelle. This could lead to increased immunogenicity as a result of the presence of multiple epitopes exposed on the micellar surface. The immune system reacts vigorously to multimeric forms of epitopes: B-cells of the immune system respond independently of T-helper cells if identical multimeric antigen epitopes are recognized (14). When several epoetin molecules are attached to micelles, identical multimeric epitopes are present that may prompt the B cells of the immune system to make antibodies.

These antibodies would also recognize the endogenous erythropoietin.

It cannot be excluded that mixed micelles of Tween 80 and (monomeric or oligomeric) epoetin contains partly unfolded or misfolded epoetin. Nevertheless, because epoetin in the micelle fractions was recognized by antibodies in the immunoassay, these fractions at least contain preserved epitopes that are also present on native epoetin. Confirming the protein structure of micelle-associated epoetin (e.g., with spectroscopic techniques) is an extremely difficult task because the epoetin content in these fractions is very small relative to the (at least 100-fold) excess of free epoetin.

It is possible that after subcutaneous administration of Eprex®/Erypo®, the small fraction of micellar epoetin is responsible for the induction of antibodies observed in some patients. After subcutaneous injection, the micelles may initially stay intact and encounter immune cells before they are diluted by body fluids. No cases have been reported associated with intravenous administration. It may be that the micelles disappear rapidly because of the immediate dilution of Tween 80 below its CMC in the bloodstream. For NeoRecormon® the Tween 20 concentration in the formulation is very close to the CMC. Therefore, on subcutaneous injection, rapid dilution by interstitial fluids makes exposure of multimeric forms to the immune system highly unlikely.

We recognize that the presence of micelle-associated epoetin as a risk factor for immunogenicity in patients at this moment remains hypothetical. Follow-up studies in which the micelle-associated epoetin is tested in animal models may shed light on the correlation between the formulation of epoetin and its immunogenicity. If such a correlation can be confirmed, ways to improve the formulation of epoetin should be studied. Lowering the surfactant concentration seems obvious, but other adjustments of the formulation might be necessary to guarantee both a sufficient shelf life and the absence of immunogenicity of epoetin.

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