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molecular pharmaceutics

Erythropoietin Produced in a Human Cell Line (Dynepo) Has Significant Differences in Glycosylation Compared with Erythropoietins Produced in CHO Cell Lines

Zahra Shahrokh,[†] Louise Royle,[‡,§] Radka Saldova,[‡] Jonathan Bones,[‡] Jodie L. Abrahams,[‡] Natalia V. Artemenko,[‡] Steve Flatman,[||] Mike Davies,[||] Alison Baycroft,[||,⊥] Surinder Sehgal,[||] Michael W. Heartlein,[†] David J. Harvey,[#] and Pauline M. Rudd*[,‡]

Shire Human Genetic Therapies, Cambridge, MA, USA, Lonza Biologics, plc, Slough, U.K., NIBRT Dublin Oxford Glycobiology Laboratory, Conway Institute, University College Dublin, Belfield, Dublin 4, Ireland, and Glycobiology Institute, Department of Biochemistry, Oxford University, Oxford, U.K.

Received October 14, 2010; Revised Manuscript Received December 6, 2010; Accepted December 7, 2010

Abstract: Recombinant human erythropoietin has been used to treat anemia associated with chronic renal disease. This paper provides a comprehensive comparative analysis of Dynepo and three other commercial erythropoiesis stimulating agents, Eprex, NeoRecormon and Aranesp. We found significant differences in the type, levels and amount of *O*-acetylation of sialic acids. Sialic acids and *O*-acetylation present provide protection from clearance from circulation. Aranesp had up to six *O*-acetyl groups attached to the sialic acids. Eprex and NeoRecormon had only minor amounts of *O*-acetylation while Dynepo had none. Dynepo had no Neu5Gc, which is potentially immunogenic for humans. Dynepo contained the least amount of disialylated and Aranesp the highest amount of tetrasialylated glycans. NeoRecormon and Eprex contained more trisialylated, but less tetrasialylated glycans than Dynepo and Aranesp. Dynepo had the highest amount of tetraantennary glycans and the lowest amounts of triantennary glycans with a β1-6-GlcNAc linkage. All the samples contained poly-*N*-acetyl-lactosamine repeats with Dynepo having the least. The major *N*-acetyl-lactosamine extensions in Dynepo and Aranesp were on biantennary glycans, whereas in NeoRecomon and Eprex they were on triantennary glycans. The sLe^x epitope was only detected in Dynepo.

Keywords: Erythropoietin; glycosylation; Dynepo; human cell line; CHO cell line

Introduction

Human erythropoietin (EPO) is a hematopoietic cytokine required for the differentiation and proliferation of precursor cells into red blood cells.[1] EPO activates cells by binding and orientating two cell surface erythropoietin receptors (EPORs) which trigger an intracellular phosphorylation cascade.[1] Erythropoietin imposes a unique orientation to its receptor that is responsible for optimal signaling through intracellular kinase pathways.[1] Recombinant human erythropoietin (rHuEPO) has been used to treat anemia associated with chronic renal disease since 1988.[2] It is also approved to treat anemia associated with other conditions such as

* Corresponding author. Mailing address: Dublin-Oxford Glycobiology Laboratory, NIBRT, Conway Institute, University College Dublin, Belfield, Dublin 4, Ireland. Phone: (+353) 1716 6728. Fax: (+353) 1716 6950. E-mail: pauline.rudd@nibrt.ie.

† Shire Human Genetic Therapies.

‡ University College Dublin.

§ Current address: Ludger Ltd, Culham Science Centre, Abingdon, Oxfordshire OX14 3EB, U.K.

|| Lonza Biologics, plc.

⊥ Current address: Oxford Nanopore Technologies Ltd, Oxford Science Park, Oxford OX4 4GA, U.K.

Oxford University.

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10.1021/mp100353a
Published on Web 12/07/2010

AIDS. While rHuEPO is considered a safe product, in rare instances it provokes antibody-induced red blood cell aplasia (PRCA). This condition has now been observed for many available recombinant EPO products, but is specifically pronounced with Eprex during subcutaneous administration.[3] During rHuEPO-induced PRCA, anti-EPO antibodies emerge that neutralize rHuEPO as well as endogenous EPO, leaving patients in an anemic state that can only be corrected through blood transfusions. While the neutralizing antibodies target the protein core of both endogenous and recombinant EPO, it has been speculated that the carbohydrate profile of recombinant EPO may, among other factors including formulation and product materials, contribute to the evolution of EPO-induced PRCA.[4,5] Highly specific anti-EPO antibodies with low-affinity binding were identified in 69% of renal failure patients and were proposed to be linked to immunogenic glycosylation epitopes.[6] Besides PRCA, the clinical response to EPO is variable. Within the renal patient population, about 10% fail to respond to rHuEPO with an associated increase in reticulocytes and hemoglobin. The reasons are poorly understood, but an underlying inflammatory disease state has been linked to EPO resistance.[7]

rHuEPO is a highly glycosylated protein with *N*-linked sites at Asn 24, Asn 38, and Asn 83 and a single *O*-linked site at Ser-126. About 40% of the molecular weight can be attributed to carbohydrate residues. Apart from Dynepo, all currently available EPO products are produced in Chinese hamster ovary (CHO) cells. CHO cells express a different set of glycosylation enzymes than human cells, consequently, the carbohydrate profiles of human and hamster cell-derived EPO are different.[8]

Darbepoetin alfa (Aranesp) is a synthetic hyperglycosylated analogue of epoetin alfa (Eprex) which has a 3.6-fold longer serum half-life than epoetin.[9−12] This erythropoiesis-stimulating protein was engineered to test the hypothesis which suggested that a few additional mutations of the amino acid content of epoetin (Ala30Asn, His32Thr, Pro87Val, Trp88Asn, and Pro90Thr) with a consequent increase of sialylated oligosaccharides would lead to an extension of the glycoprotein's terminal half-life.[10,13] The final structure of the novel erythropoiesis-stimulating protein Aranesp, which was successfully approved by FDA and EMA, contains five *N*-linked carbohydrate chains (Asn 24, Asn 30, Asn 38, Asn 83 and Asn 88, up to 22 sialic acid residues) in comparison with three *N*-glycosylation sites (up to 14 sialic acid residues) of natural erythropoietin and of the commercial erythropoiesis stimulating agents (ESAs) Eprex, NeoRecormon, and Dynepo (Figure 1).[14] The carbohydrate molecules improve its *in vivo* biological activity and increase the molecular weight of the glycoprotein from approximately 30,400 Da to 38,500 Da.

Glycosylation on recombinant human erythropoietin has been reported.[15−19] Many differences were observed between Dynepo and Aranesp especially in sialylation of *O*-glycans, heterogeneity, and absence of *N*-glycolylneuraminic acid.[16] Differences in *O*-acetylation were observed between Eprex and NeoRecormon.[18] This paper provides a comprehensive comparative analysis of Dynepo (epoetin delta) and three other ESAs: Eprex (epoetin alpha), NeoRecormon (epoetin beta), and Aranesp (darbepoetin alpha). We have evaluated the levels of *N*-glycolylneuraminic acid (Neu5Gc) and *N*-acetylneuraminic acid (Neu5Ac), sialic acid linkage (α2→3 or α2→6), *O*-acetylation of sialic acids, levels of total sialic acids, presence of sialyl Lewis x (sLe^x) epitopes, the relative proportions of mono- di-, tri- and tetraantennary structures and poly-*N*-acetyl-lactosamine repeats in all samples. Our results are in agreement with the previous reports, but we

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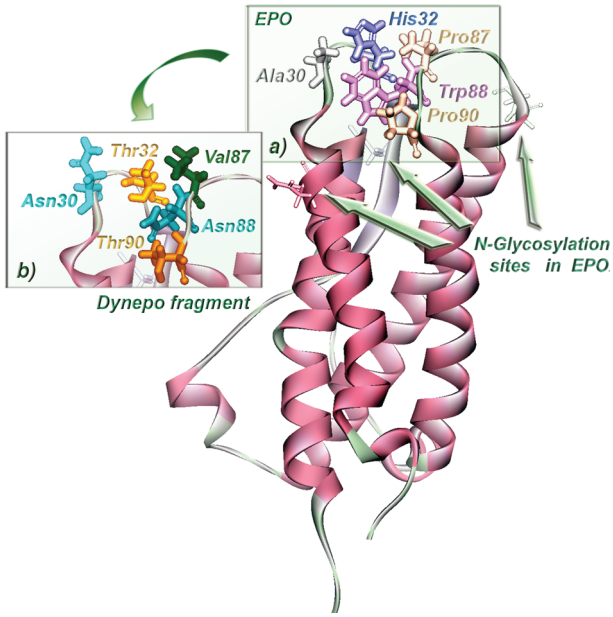

Figure 1. Structural changes in darbepoetin alfa (Aranesp) compared to epoetin alfa (Eprex). Models of both proteins are based on the 1EER crystal structure of a mutant of human erythropoietin from which the standard glycosylation sites (Asp 24, Asp 38 and Asp 83) had been removed[1] to reduce the conformational heterogeneity in the CD crossover loop. In the human erythropoietin model all three *N*-glycosylation sites were restored. In the model of the darbepoetin, two short amino acid motifs were mutated to increase the number of *N*-glycosylation sites. Both models were implemented using the "Build mutant" protocol of the MODELER[50,51] program module within Discovery Studio 2.5.[52] Differences between original "passive/inactive" motifs in EPO structure and their mutations in darbepoetin are highlighted in (a) and (b) respectively. A mutation of five amino acid residues in the EPO structure reveals two extra *N*-glycosylation sites and 50-fold increase in their activity. Three asparagine residues visualized using "stick" model within typical *N*-glycosylation sites, present in EPO, are shown using standard secondary structure color scheme. Structural differences between structural motifs in both protein structures, containing five mutated amino acids, are highlighted using Rasmol color scheme for amino acids.

have also discovered additional features of these human erythropoiesis stimulating agents.

Materials and Methods

Erythropoiesis Stimulating Agents (ESAs). Eprex (Ortho Biotech), NeoRecormon (Roche), and Aranesp (Amgen) were used. Gene-activated Dynepo was manufactured at Lonza Biologics. It was expressed by gene-activation technology in a human fibrosarcoma cell line HT-1080. A serum-free bioreactor cell culture process was used to manufacture Dynepo, and the harvested cell culture material was purified by conventional chromatographic and ultrafiltration methods. Dynepo was formulated to approximately 0.5 mg/mL in phosphate buffered saline at pH 7.0 containing polysorbate 20, and stored frozen below −65 °C until use. The Dynepo used for these studies had >99.5% purity by reversed phase HPLC and size exclusion chromatography.

***N*-Glycan Release.** The contents of one syringe from each sample consisting of 0.5 mL aliquots (0.3 mL for NeoRecormon) were dried down, then reduced and alkylated with dithiothreitol and iodoacetamide. The aliquots were then split into two and duplicates run on different days. The samples were set into SDS−PAGE gel blocks, washed, and digested with protein-*N*-glycosidase F (PNGaseF, Prozyme, San Leandro, CA, USA) overnight, the *N*-glycans were then extracted and 50% of the *N*-glycan pool was labeled with the fluorophore 2-aminobenzamide (2-AB). The details of this method are provided in references.[20,21]

Sialic Acid Analysis. Sialic acids were released and derivatized with 1,2-diamino-4,5-methylenedioxybenzene (DMB) as described earlier.[16] Briefly, sialic acids were released from erythropoietin samples by mild acid hydrolysis (2 M acetic acid, 80 °C, 2 h) or enzymatically *via* incubation with neuraminidase (Sialidase A, Prozyme, San Leandro, CA, USA). 20 μL of DMB labeling solution (DMB in mercaptoethanol and sodium dithionite, Ludger, Abingdon, U.K.) was added to a 5 μL aliquot of the release solution, and the mixture was incubated for 3 h at 50 °C in the dark. The reaction was quenched by adding 475 μL of water. The labeled samples were diluted 1/10 (or 1/100 in the case of Aranesp and the fetuin controls) and analyzed using RP-HPLC on a Waters XBridge BEH C_{18} 150 × 2.1 mm i.d., 3.5 μm column with fluorescence detection (excitation λ = 343 nm, emission λ = 448 nm).

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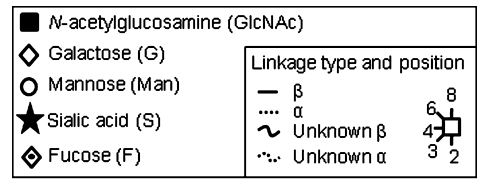
Weak Anion Exchange (WAX) HPLC. WAX-HPLC was performed using a Vydac 301VHP575 7.5 × 75 mm column (Anachem, Luton, Bedfordshire, U.K.) on a 2695 Alliance separations module with a 474 fluorescence detector (Waters, Milford, MA, USA). Solvent A was 0.5 M formic acid adjusted to pH 9.0 with ammonia solution, and solvent B was 10% (v/v) methanol in water. Gradient conditions were as follows: a linear gradient of 0 to 5% A over 12 min at a flow rate of 1 mL/min, followed by 5 to 21% A over 13 min and then 21 to 50% A over 25 min, 80 to 100% A over 5 min, and then 5 min at 100% A. Samples were injected in water. A fetuin *N*-glycan standard was used for calibration.[20] All HPLC units were equipped with Waters temperature control modules and Waters 2475 fluorescence detectors set with excitation and emission wavelengths of 330 and 420 nm, respectively.[21]

Hydrophilic Interaction Liquid Chromatography (HILIC). HILIC was performed using a TSK-Gel Amide-80 4.6 × 250 mm column (Anachem) on a 2695 Alliance separations module (Waters) equipped with a Waters temperature control module and a Waters 2475 fluorescence detector. Solvent A was 50 mM formic acid adjusted to pH 4.4 with ammonia solution. Solvent B was acetonitrile. The column temperature was set to 30 °C. Gradient conditions were a linear gradient of 20−58% A, over 152 min at a flow rate of 0.4 mL/min.[21] Samples were injected in 80% acetonitrile.[20] Fluorescence was measured at 420 nm with excitation at 330 nm. The system was calibrated using an external standard of hydrolyzed and 2-AB-labeled glucose oligomers to create a dextran ladder, as described previously.[20]

Exoglycosidase Digestions. All enzymes were purchased from Prozyme (San Leandro, CA, USA). The 2-AB-labeled glycans were digested in 10 μL of 50 mM sodium acetate buffer, pH 5.5 for 18 h at 37 °C, using arrays of the following enzymes at the indicated concentrations: ABS, *Arthrobacter ureafaciens* sialidase (EC 3.2.1.18), 1 U/mL; NAN1, *Streptococcus pneumoniae* sialidase (EC 3.2.1.18), 1 U/mL; BTG, bovine testes beta-galactosidase (EC 3.2.1.23), 1U/mL; SPG, *Streptococcus pneumoniae* β-galactosidase (EC 3.2.1.23), 0.1 U/mL; BKF, bovine kidney alpha-fucosidase (EC 3.2.1.51), 1 U/mL; AMF, almond meal alpha-fucosidase (EC 3.2.1.111), 3 mU/mL; GUH, β-*N*-acetylglucosaminidase cloned from *S. pneumonia*, expressed in *Escherichia coli* (EC 3.2.1.30), 4 U/mL. After incubation, enzymes were removed by filtration through a protein-binding EZ filters (Millipore Corporation, Billerica, MA, USA).[20] *N*-Glycans were then analyzed by HILIC or WAX-HPLC.

***N*-Glycan Nomenclature.** We have used "Oxford notation" nomenclature according to Royle et al.[21] (linear notation) and Harvey et al.[22] (symbolic notation) (Figure 2). All *N*-glycans have two core GlcNAcs; F at the start of the abbreviation indicates a core α1→6 fucose linked to the inner GlcNAc; M*x*, number (*x*) of mannose residues on core GlcNAcs; A*x*, antenna number. Thus, A2 defines a biantennary glycan with both GlcNAcs β1→2 linked; A3 is a triantennary glycan with a GlcNAc linked β1→2 to both mannose residues and the third GlcNAc linked β1→4 to the α1→3 linked mannose; A3′ is a triantennary with a GlcNAc linked β1→2 to both mannose residues and the third GlcNAc linked β1→6 to the α1→6 linked mannose; A4, GlcNAcs linked as A3 with an additional GlcNAc β1→6 linked to α1→6 mannose; G*x*, number (*x*) of β1→4-linked galactose residues on the antennae; F*x*, number (*x*) of linked fucose on the antenna, (3) after the F indicates that the fucose is α1−3 linked to the GlcNAc; S*x*, number (*x*) of sialic acids linked to galactose; the numbers 3 or 6 in parentheses after S indicate whether the sialic acid has an α2−3 or α2−6 linkage.



Diagrammatic nomenclature of glycans.
Each monosaccharide is represented by a distinct shape and filled to indicate *N*-acetylation at the non-reducing terminus.

Figure 2. Monosaccharide residue symbols and bond angles of the pictured glycans.

Mass Spectrometry. 1 μL of each unlabeled sample was placed on a Nafion 117 membrane] (floating on water) for 30 min to remove any background contamination.[23] The sample was diluted 1:6 in MeOH:H_2O (1:1, v:v) containing a trace of ammonium phosphate to convert any neutral glycans to phosphate adducts and infused with Proxeon (now part of Thermo Fisher Scientific), Odense, Denmark) nanospray capillaries into a Waters-Micromass Q-Tof Ultima Global mass spectrometer for acquisition of MS and MS/MS spectra. Spectra were smoothed (Savitsky−Golay 5 × 2). Compounds were identified by negative ion MS/MS both before and after desialylation with *Arthrobacter ureafaciens* sialidase.[24−27]

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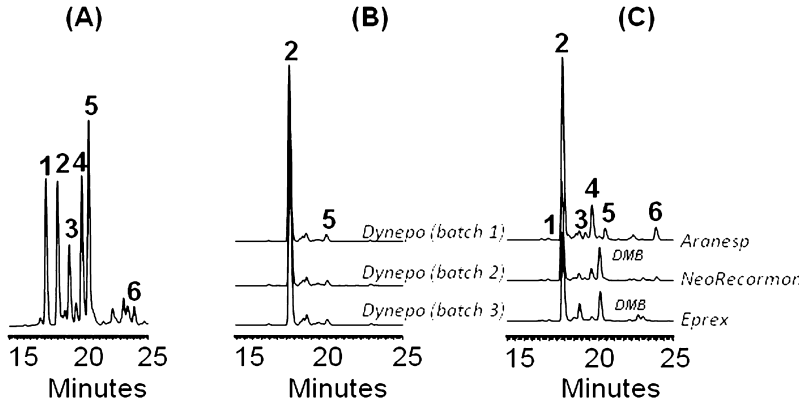

Peak identification: **1** *N*-glycolylneuraminic acid, **2** *N*-acetylneuraminic acid, **3** 5,7-diacetylneuraminic acid, **4** 5-glycolyl-9-acetylneuraminic acid, **5** 5,9-diacetylneuraminic acid and **6** 5-7/8-9-triacetylneuraminic acid.

Figure 3. (A) Gradient separation of a standard mixture of DMB labeled sialic acids on a Waters XBridge BEH C18 2.1 × 15 cm, 3.5 μm column using 10 mM ammonium formate pH 4.4 and acetonitrile. (B) Analysis of 3 batches of Shire Dynepo showed that the product contained only *N*-acetylneuraminic acid with a small proportion of 5,9-diacetylneuraminic acid. (C) Speciation studies showed that the other erythropoietin samples contained both *N*-acetyl and *N*-glycolyl sialic acids.

Results

The scope of this study was to analyze the *N*-glycan structures released from a range of commercial ESAs, paying particular attention to the linkage type, level, and *O*-acetylation of sialic acids; presence of sLe^x epitopes; the relative proportions of mono-, di-, tri- and tetraantennary structures; and poly-*N*-acetyllactosamine repeats.

Levels of Neu5Ac and Neu5Gc sialic acids were determined following release by acid hydrolysis. Mass spectrometry was used to detect *O*-acetylation as well as Neu5Ac and Neu5Gc sialic acids. The relative levels of sialylation on the *N*-glycans from the different samples were calculated from the WAX-HPLC[28] and hydrophilic interaction liquid chromatography (HILIC) chromatograms. *N*-Glycans, analyzed by HILIC coupled with exoglycosidase digestions, provided sensitive detection (the limit of detection (LOD) was 2 fmol).

Determination of Sialic Acid Forms (Neu5Ac vs Neu5Gc). The levels of Neu5Ac and Neu5Gc were determined for each sample by acid hydrolysis, selective fluorescent labeling of the released sialic acids and subsequent profiling via RP-HPLC.

The RP-HPLC separation of Neu5Ac and Neu5Gc is shown in Figure 3A. In order to assess the presence or absence of Neu5Gc across various ESA products, the LOD was experimentally determined using the Neu5Gc and Neu5Ac reference standards. LOD was defined as the lowest concentration resulting in a visible chromatographic peak above the baseline noise. The experimentally determined LODs for Neu5Ac and Neu5Gc were 0.012 nmol and 0.006 nmol, respectively.

Table 1. The Total Sialic Acid Content in Samples

	Dynepo	NeoRecormon	Eprex	Aranesp
nmol of Neu5Ac/ nmol of protein	13.40	12.50	10.48	18.88
nmol of Neu5Gc/ nmol of protein	nd[a]	0.14	0.15	0.22
% Neu5Gc	0	1.10	1.40	1.10

[a] Not detected.

No Neu5Gc was detected when 10 μg of epoetin delta (Dynepo) was analyzed (Figure 3B); Neu5Gc was readily detectable when 10 μg of NeoRecormon, Eprex and Aranesp were assayed (Figure 3C). Quantitative analysis of these data indicates that the Neu5Gc portion of the total sialic acid content for these samples of NeoRecormon, Eprex, and Aranesp was 1.1%, 1.4% and 1.1% (by mol), respectively (Table 1). The Neu5Ac and Neu5Gc contents across the various recombinant EPO products are listed in Table 1. Dynepo was the only product without quantifiable levels of Neu5Gc using the standard validated method for sialic acid determination by acid hydrolysis, as would be expected in a human expression system.

Sialic acids were also released from Dynepo by enzymatic desialylation to further confirm that the Neu5Gc content of Dynepo is below the limit of quantification. The results were comparable to those obtained by acid hydrolysis for both the Neu5Ac and the Neu5Gc analysis confirming that the sialic acid release procedure does not influence the determined value for Neu5Ac and Neu5Gc content.

The results suggest that Neu5Gc is present and readily detectable in NeoRecormon, Eprex and Aranesp, with

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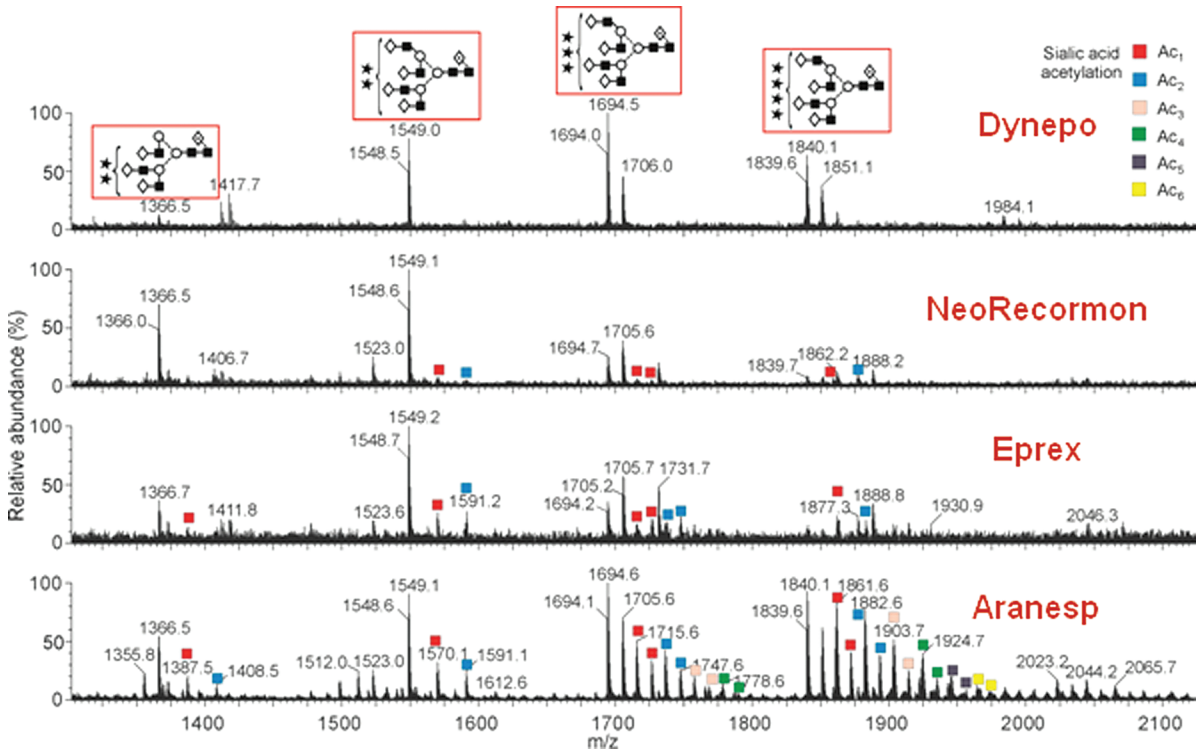

Figure 4. Summary ESI-MS spectra showing acetylation of sialic acids in all products except Dynepo.

Aranesp containing the highest amount of Neu5Gc per nmole product and Eprex the highest relative amount of Neu5Gc per total sialic acid, but Neu5Gc is below the limit of detection for Dynepo.

Analysis of Sialic Acid Linkages. The charged fractions of samples, collected by WAX, were treated with sialidases specific for α2−3,6,8 and α2−3,8 sialic acid. No differences were detected between HILIC chromatograms following digestion of the total *N*-glycan pool with sialidase of different specificity (NAN1, which is specific for α2−3 and α2−8 sialic acids, or ABS which removes all sialic acids), indicating that all of sialic acids are α2−3 linked to Gal (or α2−8 linked in polysialic acids). Where a peak in the tri- or tetrasialylated fractions digested to a biantennary glycan, the extra sialic acid could be α2−8 linked to the α2−3 sialic acid linked to galactose. No α2−6 linked sialic acids were identified by either HILIC as assessed by exoglycosidase digestions (data not shown) or ESI-MS/MS fragmentation (data not shown).

***O*-Acetylated Sialic Acids.** Figure 4 shows the negative ion ESI-MS spectra for all four samples. The spectra of Eprex, NeoRecormon and particularly Aranesp glycans contained ions spaced by 42 mass units, consistent with the presence of *O*-acetyl groups as reported earlier.[16,17] Negative ion CID spectra showed that these *O*-Ac groups were located on the sialic acids and that both mono- and di-*O*-acetyl-substituted sialic acids were present. The substitution positions of these *O*−Ac groups were determined by RP-HPLC. Aranesp has abundant *O*-acetylation on its sialic acids, while Eprex and NeoRecormon have minor amounts and Dynepo has no *O*-acetylation. Aranesp contains up to six *O*-acetyl groups on the Neu5Ac residues.

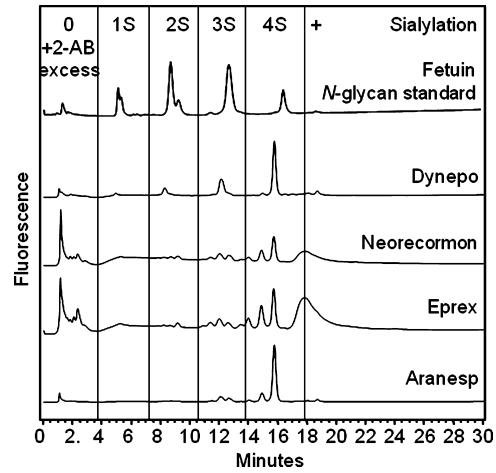

Figure 5. WAX profiles of samples with fetuin *N*-glycans as a reference standard. Glycans eluted according to the number of sialic acids they contained. The broad peaks in >4S of NeoRecormon and Eprex did not contain any glycans and were therefore due to other contaminating components.

Relative Levels of Mono-, Di-, Tri-, Tetra- and Poly-sialylated Glycans. The labeled glycans were separated into pools according to charge by WAX-HPLC. The glycans contained mostly from zero to four sialic acids (Figures 5 and 6). Assignments of charge were made by comparison with a standard of fetuin *N*-glycans. Aranesp had the highest amount of tetrasialylated structures (63.9%), Dynepo contained the least amount of disialylated structures (5.4%) (Table 2). NeoRecormon and Eprex had more trisialylated structures and less tetrasialylated structures than the others

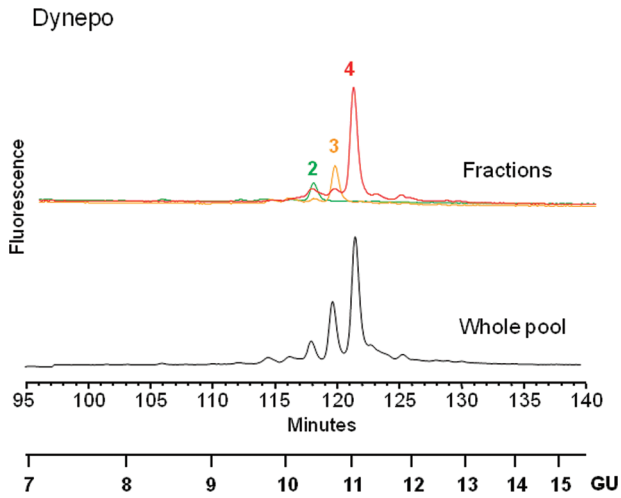

Figure 6. Dynepo *N*-glycan fractions separated by charge collected from WAX-HPLC, run on HILIC. Traces from different charge pools are overlaid: di-sialylated, green; tri-sialylated, orange; tetra-sialylated, red.

Table 2. The Relative Amounts of Sialylation between Samples Calculated from the WAX-HPLC and HILIC Results[a]

	% area[b]			
no. of sialic acids	Dynepo	NeoRecormon	Eprex	Aranesp
S1	0.85	1.90	0.00	0.00
S2	5.39	11.65	10.89	8.85
S3	31.41	35.43	32.21	23.31
S4	62.35	51.01	53.40	63.92
polysialic acid[c]	17.29	16.34	24.94	26.26

[a] Structures with disialic acids were calculated as % of total structures from HILIC. [b] The relative amounts of sialylation between samples were calculated from the HILIC results listed in Table 3. No significant amounts of neutral glycans were detected in Dynepo and NeoRecormon following separation into charged fractions by WAX. Eprex contained 3.48% and Aranesp 3.68% neutral glycans. [c] Polysialic acids or other sialic acids in addition to one on each terminal glucose.

(Table 2). Eprex and Aranesp had higher amount of glycans with additional sialic acids (i.e., more sialic acids than galactoses) than Dynepo and NeoRecormon (Table 2).

The differently charged pools were further separated by HILIC (Dynepo example Figure 6); the relative levels of sialylation in the different samples were calculated from the WAX-HPLC and HILIC data and are listed in Table 2.

HILIC and Exoglycosidase Digestions. The *N*-glycans from Dynepo, Aranesp, Eprex and NeoRecormon were analyzed using HILIC coupled with exoglycosidase digestions (Figure 7). The released *N*-glycan pools were digested with sialidase, β-galactosidase, fucosidase (BKF to remove core α1−6 fucose and AMF to remove outer arm α linked fucose) and *N*-acetyl-glucoseaminidase. Ten times more sialidase than usually required was added to ensure complete removal of all sialic acids including *O*-acetylated sialic acids, as it has been reported that the sialidase has reduced activity

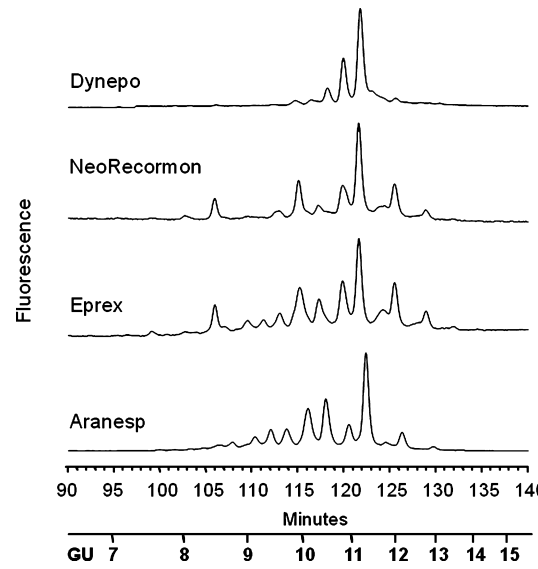

Figure 7. HILIC profiles of Dynepo, Aranesp, Eprex and NeoRecormon.

on these substrates.[29] The data are summarized in Table 3. A total of 27, predominantly sialylated structures were identified with only very low amounts of neutral or mono-sialylated glycans detected in each sample.

Figures 8A and 8B show HILIC chromatograms of the digestion profiles of Dynepo, annotated with a graphical representation of the major glycan present to illustrate which monosaccharides are removed from the nonreducing end of one particular glycan following the action of specific exoglycosidase. HILIC chromatograms are presented on two different scales: normalized to the highest peak (Figure 8A) and magnified to view the smaller peaks (Figure 8B).

From a combination of the exoglycosidase digestions and the MS/MS data we conclude that the core neutral glycans in these samples are a mixture of biantennary, triantennary (with both the β1−4 and β1−6 isoforms present) and tetraantennary structures, which can also contain one or two *N*-acetyllactosamine extensions. Digestions of the total *N*-glycan pool with galactosidases of different specificity (SPG, which is specific for β1→4Gal, or BTG which removes all βGal) produced identical results, indicating that all of the galactose residues were β1→4 linked.

Core Glycan Structures. There are major differences in the relative amounts of the different core structures between Dynepo and the other samples detected by HILIC with exoglycosidase sequencing (Table 4). Dynepo has the highest level of tetraantennary structures (65% vs 28 to 44%). The other ESAs have high amounts of triantennary structures with a β1→6 GlcNAc linkage (A3′) (12 to 14% vs 3% in Dynepo) in addition to the more common β1→4 GlcNAc linkage. The major poly-*N*-acetyllactosamine extensions in Dynepo and Aranesp were on biantennary glycans, whereas in Neorecomon and Eprex they were on

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Table 3. Summary of the Structures Identified

Structure	GU	Dynepo	Neorecormon	Eprex	Aranesp
		%Area			
FA2G2	7.56	-	-	0.78	0.20
?	7.70	-	-	-	0.25
FA2G2 isomer?	7.80	-	-	1.36	0.40
FA2G2S1	7.96	-	0.96	-	-
?neutral	8.12	-		-	0.63
FA2G2S2	8.38	0.30	5.42	4.85	2.01
FA3G3	8.62	-	-	1.34	2.45
FA2G2S3	8.91	0.22	2.21	3.55	3.14
?S3/4	9.12	-	-	-	2.15
FA3'G3S1	9.13	-	0.15	-	-
FA3G3S2	9.36	-	2.93	2.63	6.03
FA3G3S3	9.39	0.88	0.52	4.86	7.37
FA3'G3S2	9.60	0.92	-	-	0.44
FA3'G3S3	9.75	1.47	10.73	7.07	3.35
FA3'G3S4	9.77	0.68	1.27	4.81	10.39
FA2G2Lac1aS3	9.99	2.51	-	-	0.43
FA2G2Lac1bS2	10.03	0.35	-	-	-
FA4G4S1	10.05	0.85	0.27	-	-
FA2G2Lac1aS4	10.10	3.52	3.86	6.35	14.87
FA4G4S2	10.33	3.81	2.09	0.99	0.37
FA2G2Lac1bS3	10.33	1.20	-	1.50	-
FA2G2Lac1bS4	10.55	6.93	-	3.58	6.17
FA4G4S3	10.64	13.73	13.40	8.70	3.20
?S1 *low*	11.01	-	0.52	-	-
FA4G4S4	11.03	41.53	27.74	21.10	25.06
FA4F1G4S3	11.06	2.68	-	-	-
FA4F1G4S4	11.20	2.79	-	-	-
FA3G3Lac1S2	11.29	-	1.20	2.42	-
FA3G3Lac1S3	11.43	7.55	6.66	4.62	3.60
FA3G3Lac1S4	11.70	5.39	12.87	11.50	6.14
FA4G4Lac1S3	12.30	1.18	1.91	1.92	0.07
FA4G4Lac1S4	12.40	0.76	4.45	4.73	1.29
?S3/4	12.86	0.74	0.84	1.34	-

The relative areas of glycans within coeluting peaks have been calculated from the WAX fractionation results. Several of these peaks overlap. The individual % areas in these peaks have been calculated from the relative amounts in the different WAX fractions. Where Lac1 was found as two different isomers (on [3] and [6] arm), they are referred to as isomers a and b, respectively.

triantennary glycans (Table 4). The presence of the two isomers was confirmed by MS/MS; the E-type ion at *m/z* 831 was diagnostic for the A3 structure whereas the presence of the D, $[D - 18]^-$ and $[D - 36]^-$ ions at 1053, 1035, and 1017 respectively[30] showed the presence of the other isomer (data not shown). The full list of structures identified with their relative amounts is given in Table 3. Some minor components were not fully characterized and are labeled as such.

Outer-Arm Fucosylation (sLe^x). The only sample with detectable amounts of outer arm fucosylated glycans was Dynepo where fucose was detected attached to the tetraantennary glycan. In the HILIC profiles of Dynepo, structures containing this epitope represented between 1% and 2% of the total glycans. The presence of this outer arm fucosylated structure is highlighted in Figure 8B where the digestion sequence segregates the peaks containing an outer arm fucose. The Dynepo sample is the only one with clear peaks above background. The fucose is also attached to a GalNAc (as it hinders removal of the galactose until after digestion of the fucose, Figure 8B) and is therefore α1→3 linked, as the 4 position is occupied by the galactose. As all of the glycans were sialylated in the undigested pool, this fucose formed a sialyl Lewis x (sLe^x) epitope in the Dynepo samples.

Discussion

Neu5Gc Is Present on ESAs Produced by CHO Cells but Not on Dynepo from Human Cell Line. As Dynepo is the first recombinant EPO product generated in human cells, we evaluated the potential presence of Neu5Gc. We have shown that recombinant human EPO generated in hamster cells contains approximately 1% to 1.4% of Neu5Gc residues relative to the total sialic acid content. This is consistent with prior publications describing between 1% and 3% Neu5Gc in hamster-cell derived rHuEPO and lower than the 7% reported for recombinant granulocyte-macrophage colony-stimulating factor,[19,31,32] and may be dependent on the batches of rHuEPO materials tested. By contrast, Dynepo generated through gene-activation in human cells contains no Neu5Gc residues as confirmed by two independent

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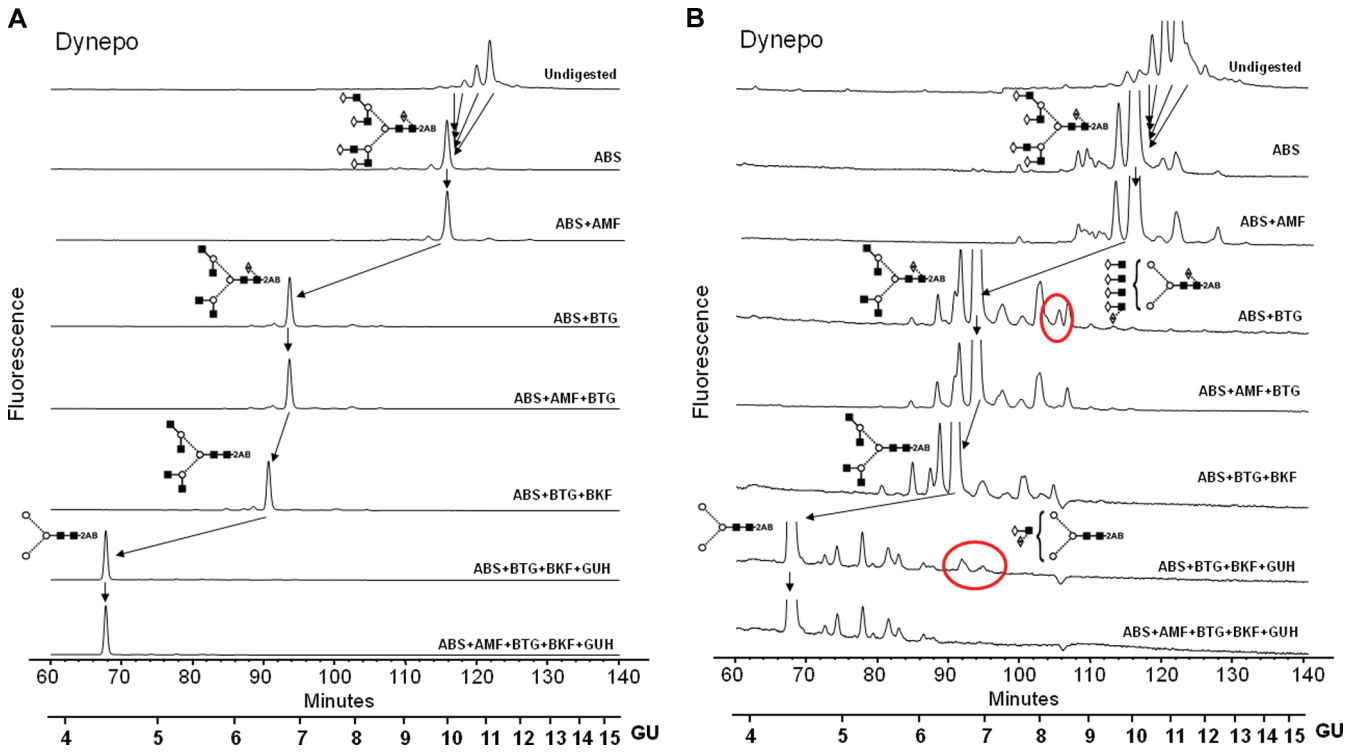

Figure 8. HILIC profiles of Dynepo before and after digestion with a range of exoglycosidases (see Materials and Methods for abbreviations). (A) The digestion pathway of the core fucosylated tetraantennary structure is shown. The structure is found in the undigested pool as mono- di-, tri- and tetrasialylated analogues. (B) HILIC profiles are zoomed in to highlight small peaks. The peaks circled in red move following digestion with AMF, indicating that they contain an outer arm fucose which originated from a sialyl Lewis x structure.

Table 4. The Relative Amounts of the Different Core Structures

	% area			
core structure	Dynepo	Neorecormon	Eprex	Aranesp
not identified	0.00	0.52	1.36	3.42
FA2	0.52	8.58	9.18	5.34
FA3	0.88	3.45	8.83	15.85
FA3′	3.07	12.15	11.88	14.19
FA4	65.40	43.50	30.79	28.63
FA2Lac	14.51	3.86	11.43	21.46
FA3Lac	12.94	20.73	18.54	9.74
FA4Lac	2.68	7.20	7.99	1.36
total PolyLac	30.13	31.78	37.95	32.57

methods to release sialic acid from the protein, and it is consistent with a previous report.[16]

The most common sialic acids are Neu5Ac, Neu5Gc and *N*,9-diacetyl-acetylneuraminic acid.[33] The loss of the ability to generate Neu5Gc residues during evolution is linked to brain development in humans as well as to susceptibility and resistance toward certain microbial pathogens.[34,35] Small amounts of Neu5Gc, however, are present in humans, mostly in human tumor cells but also in normal human epithelial cells and several organs.[36] Neu5Gc is taken up through food, predominantly red meat and dairy products.[36,37] The Neu5Gc residue has become variably immunogenic for humans with many humans having circulating antibodies against Neu5Gc.[37−39] Neu5Gc residues expressed on cells can potentially mediate acute rejection of these cells by human serum.[37−40] Immunogenicity and

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increased clearance of therapeutic glycoproteins containing Neu5Gc due to anti-Neu5Gc antibodies has been described.[41] "Serum-sickness" in response to intravenous administration of equine antithymocyte globulin therapy has been linked to the Neu5Gc residues present in these molecules.[42,43] Collectively, these findings suggest that circulating Neu5Gc antibodies can attack Neu5Gc-expressing cells and proteins. Human sera containing high levels of these anti-Neu5Gc antibodies deposit complement on Neu5Gc-expressing cells.[37] The binding of these antibodies results in complement-mediated cytotoxicity.[37] rHuEPO expressing Neu5Gc, therefore, could be destroyed by complement-mediated cytotoxicity in patients with high levels of anti-Neu5Gc antibodies.

A frequent complication of EPO-treatment in patients with chronic renal disease is EPO-resistance, defined as failure to respond to even high doses of recombinant erythropoietin with appropriate increase in reticulocytes and hemoglobin. Circulating anti-Neu5Gc antibodies may potentially contribute to EPO-resistance by binding and incapacitating recombinant EPO carrying Neu5Gc residues.

ESAs Contain Different Amounts of Sialic Acids That Are *O*-Acetylated Except in Dynepo. Aranesp has the highest amount of total sialic acids, followed by Dynepo and NeoRecormon with Eprex having the least (Table 1). This is not surprising, as Aranesp is a synthetic hyperglycosylated analogue of Eprex which has a 3.6-fold longer serum half-life than epoetin[9−11] due to two additional *N*-glycosylation sites.[12] We also detected more sialic acid residues than galactose suggesting the presence of α2,8-linked sialic acid chains in Eprex and Aranesp than Dynepo and NeoRecormon. Polysialic acid (PSA) is heavily negatively charged, and it can thereby inhibit the interaction of other cell adhesion molecules by effectively repelling cell membranes of neighboring cells.[44] Only α2→3 linked sialic acids were identified linked to galactose in all ESAs. Glycans terminating in α2→3 linked sialic acid can act as binding ligands for selectins,[44] and both α2→3 and α2→6 linked sialic acid protect glycoconjugates from removal from circulation by the asialoglycoprotein receptor.[44] Therefore, higher amounts of sialic acid could help to prolong ESA halftime in circulation.

We found abundant *O*-acetylation on the sialic acids in Aranesp, whereas only minor amounts of *O*-acetylation were detected on Eprex and NeoRecormon, with no significant amounts on Dynepo. This is a major difference between Dynepo and the other ESAs. Eprex was reported to have a higher degree of *O*-acetylation than NeoRecormon,[18] which is consistent with our data. *O*-Acetylation is one of the most common modifications on sialic acids.[45] The metabolism of acetylated sialic acids is under the control of two groups of enzymes, *O*-acetyl transferases and 9-*O*-acetyl esterases.[45] Acetylation of sialic acid is present on the outermost part of the carbohydrate moiety of membrane and secreted glycoproteins, outside the cells which explains why this modification is involved in cell−cell interactions and the nonimmune protection of mucosae.[45] *O*-Acetylation has important biological significance on the glycoprotein molecule; it increases its hydrophobicity[46] and decreases susceptibility to sialidases,[29,46−48] which could also contribute to longer half-life in the circulation for ESAs with *O*-acetylation.

ESAs Have Different Levels of Poly-*N*-acetyl-lactosamine Extensions, and Dynepo Contains Glycans with SLe^x Epitope. All the samples contained poly-*N*-acetyl-lactosamine repeats on some glycans. Dynepo had fewer of these extended antennae compared to the others. Poly-*N*-acetyl-lactosamine chains are recognized by mammalian S-type lectins (galectins), and they often terminate by sialylation or fucosylation and present these terminal glycans for interaction with lectin-like receptors for example selec-

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tins.[44] Detection of two isomers of the triantennary glycans in EPO from CHO cells has been reported before,[15] however, Llop et al. report that all the triantennary glycans in Dynepo have the A3′ structure[16,17] whereas our data show a mixture of isomers A3 and A3′.

All *N*-glycans present on the examined ESAs were found to be core fucosylated.[15] Dynepo has the highest amount of core fucosylated tetraantennary structures and was the only one of the ESAs to contain outer-arm fucosylation in the form of the sLe^x epitope. The presence of this epitope on ESAs has not been reported previously.

A recent study by McVicar et al.[49] describes the different angiogenetic potential of Dynepo, Aranesp and NeoRecormon in a mouse model of ischemia-induced retinopathy. These differences could be caused by the different glycosylation of these ESAs.

Summary

The types and amounts of the following features of glycosylation are important for the pharmacokinetics and safety of biopharmaceuticals: sialylation; the presence of *N*-glycolyl neuraminic acid; core glycan structures; polylactosamine extensions and Lewis epitopes.

Aranesp has the highest amount of tetrasialylated structures. Dynepo contained the least amount of disialylated structures and the least amount of glycans with additional sialic acids (i.e., more sialic acids than galactoses). NeoRecormon and Eprex have more trisialylated structures and less tetrasialylated structures than the others. Sialic acids were α2−3-linked to galactose with no α2−6-sialic acids detected.

A major difference between Dynepo and the other samples was the lack of *O*-acetylated sialic acids, and the lack of Neu5Gc. Aranesp had abundant *O*-acetylation on its sialic acids (with up to six *O*-acetyl groups), while Eprex and NeoRecormon had minor amounts and Dynepo had no *O*-acetylation. There was no Neu5Gc detected in Dynepo, whereas the proportion of Neu5Gc as a percentage the total sialic acid content was 1.1% for NeoRecormon, 1.4% for Eprex, and 1.1% for Aranesp.

Dynepo has the highest amount of core fucosylated tetraantennary structures (65% vs 28 to 44%). The other samples have high amounts of triantennary structures with a β1−6-GlcNAc linkage compared to Dynepo (12 to 14% vs 3% in Dynepo). All the samples contained poly-*N*-acetyllactosamine repeats on some glycans. Dynepo had less of these repeats (30%) compared to the others (32 to 38%). The major poly-*N*-acetyl-lactosamine extensions in Dynepo and Aranesp were on biantennary glycans, whereas in Neorecomon and Eprex they were on triantennary glycans. Outer-arm fucosylation (sLe^x epitope) was only detected in Dynepo where structures containing this epitope represented between 1% and 2% of total glycans.

Sialic acids and their *O*-acetylation protect ESAs from removal from circulation by the asialoglycoprotein receptor in liver.

Abbreviations Used

2-AB, 2-aminobenzamide; ABS, *Arthrobacter ureafaciens* sialidase; AIDS, aquired immune deficiency syndrome; AMF, almond meal alpha-fucosidase; Anti-Id, anti-idiotype antibody; Aranesp, darbepoetin alpha; BKF, bovine kidney alpha-fucosidase; BSA, bovine serum albumin; BTG, bovine testes β-galactosidase; CHO, Chinese hamster ovary; CID, collision-induced dissociation; DMB, 1,2-diamino-4,5-methylenedioxybenzene; DTT, dithiothreitol; Dynepo, epoetin delta; EMA, European Medicines Agency; EPO, erythropoietin; EPORs, erythropoietin receptors; Eprex, epoetin alpha; ESAs, erythropoiesis stimulating agents; ESI, electrospray ionization; FDA, Food and Drug Administration; Gal, galactose; GlcNAc, *N*-acetylglucosamine; GU, glucose unit; GUH, β-*N*-acetylglucosaminidase cloned from *S. pneumoniae*, expressed in *Escherichia coli*; HILIC, hydrophilic interaction liquid chromatography; HPLC, high performance liquid chromatography; IL1, interleukin 1; LOD, limit of detection; MS, mass spectrometry; NAN1, *Streptococcus pneumoniae* sialidase; NeoRecormon, epoetin beta; Neu5Ac, *N*-acetylneuraminic acid; Neu5Gc, *N*-glycolylneuraminic acid; N-CAM, neural cell adhesion molecule; NP-HPLC, normal phase HPLC; PAGE, polyacrylamide gel electrophoresis; PNGase F, protein *N*-glycosidase F; PRCA, antibody-induced red blood cell aplasia; PSA, polysialic acid; Q, quadrupole; rHuEPO, recombinant human erythropoietin; RP, reversed phase HPLC; SDS, sodium dodecyl sulfate; SPG, *Streptococcus pneumoniae* β-galactosidase; sLe^x, sialyl Lewis x; TNFα, tumor necrosis factor α; Tof, Time-of-flight; WAX, weak anion exchange chromatography.

Acknowledgment. The authors would like to thank Dr. Weston Struwe for careful reading of this manuscript. J.B. and P.M.R. also acknowledge the European Union FP6 GLYFDIS research program grant reference 037661 for support.

MP100353A

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