Carbohydrate Structure of Erythropoietin Expressed in Chinese Hamster Ovary Cells by a Human Erythropoietin cDNA*

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The proper glycosylation of erythropoietin is essential for its function in vivo. Human erythropoietins were isolated from Chinese hamster ovary cells transfected with a human erythropoietin cDNA and from human urine. Carbohydrate chains attached to these proteins were isolated and fractionated by anion-exchange high performance liquid chromatography (HPLC) and HPLC employing a Lichrosorb-NH₂ column. The structures of fractionated saccharides were analyzed by fast atom bombardment-mass spectrometry and methylation analysis before and after treatment with specific exoglycosidases.

Both erythropoietins were found to contain one O-linked oligosaccharide/mol of the proteins, and its major component was elucidated to be NeuNAcα₂⁺Galβ1→3(GalNAcα2→3Galβ1→3(NeuNAcα2→3Galβ1→3GalNAcOH (where NeuNAc represents N-acetylneuraminic acid) in both proteins. The N-linked saccharides of recombinant erythropoietin were found to consist of biantennary (1.4% of the total saccharides), triantennary (10%), triantennary with one N-acetyllactosaminyl repeat (3.5%), tetraantennary (31.8%), and tetraantennary with one (32.1%), two (16.5%), or three (4.7%) N-acetyllactosaminyl repeats. All of these saccharides are sialylated by 2→3-linkages. Tetraantennary with or without polylactosaminyl units are mainly present as disialosyl or trisialosyl forms, and these structures exhibit the following unique features. α2→3-Linked sialic acid and N-acetyllactosaminyl repeats are selectively present in the side chains attached to C-6 and C-2 of 2,6-substituted α-mannose and C-4 of 2,4-substituted α-mannose.

We have also shown that the carbohydrate moiety of urinary erythropoietin is indistinguishable from recombinant erythropoietin except for a slight difference in sialylation, providing the evidence that recombinant erythropoietin is valuable for biological as well as clinical use.

Erythropoietin is a glycoprotein which stimulates proliferation and differentiation of erythroid precursor cells to more mature erythrocytes (1). Erythropoietin is primarily produced in adult kidney and fetal liver cells (2–4). Patients with chronic renal failure are anemic as a result of impaired renal function which leads to a decreased production of erythropoietin (5).

Thus, availability of purified erythropoietin in quantity is essential to understand molecular mechanisms of erythropoiesis and for treatment of anemia. However, this has been hampered by the fact that only a very small amount of erythropoietin is present in starting sources, even in such cases as the urine of aplastic anemia patients (6).

In order to overcome this problem, cDNA clones for human erythropoietin have been isolated in several laboratories, and the expression of erythropoietin cDNA clones has been achieved (7–9). Furthermore, the recombinant erythropoietin has been successfully used to reverse the anemia of patients with end-stage renal disease (10, 11). Interestingly, the erythropoietin produced in Escherichia coli or yeast was inactive or very weakly active in vivo. On the other hand, the erythropoietin produced in COS cells or Chinese hamster ovary cells was found to be fully active in vivo. In agreement with these results, it has been reported that desialylation of partially purified erythropoietin results in inactivation of erythropoietin activity (12–15). Thus, it is apparent that the proper glycosylation is essential for erythropoietin activity in vivo.

These results prompted us to analyze carbohydrate structures of erythropoietin produced by transfection of recombinant DNA into Chinese hamster ovary cells. In addition, we compared those structures with carbohydrate units present in erythropoietin purified from human urine.

EXPERIMENTAL PROCEDURES

Erythropoietin—Chinese hamster ovary cells (dihydrofolate reductase*) were transfected with an expression vector which harbors the human erythropoietin cDNA as described (7). This expression vector also contains dihydrofolate reductase minigene so that stable transfected can be grown in the presence of methotrexate (16). Erythropoietin was purified from the spent medium of those cells as described (7). The purification procedure was slightly modified from that of Miyake et al. (6), and fractionation on a Vydac C₁₈ reverse-phase HPLC column (The Separations Group) was included (7). This erythropoietin will be called recombinant erythropoietin hereafter. Erythropoietin was also purified from the urine of aplastic anemia patients according to Miyake et al. (6), with a similar modification as for purification of recombinant erythropoietin. The erythropoietin purified from urine will be called urinary erythropoietin hereafter. These erythropoietin samples were prepared by Chugai Pharmaceutical Co., Ltd. (Tokyo).

Isolation of N-Linked Glycopeptides and O-Linked Oligosaccharides from Erythropoietins—Glycopeptides were prepared by Pronase digestion of erythropoietin (5 mg of recombinant erythropoietin and

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1 The abbreviations used are: HPLC, high performance liquid chromatography; FAB-MS, fast atom bombardment-mass spectrometry; Hex, hexose; HexNAc, N-acetylhexosamine; NeuNAc, N-acetylneuraminic acid; Lac, N-acetyllactosaminyl repeat.
Fractionation of Neutral Oligosaccharides by HPLC—The glycophosphate fraction containing N-linked saccharides was digested with Flavobacterium meningosepticum N-glycanase (peptide-N-N-(N-acetyl-
\beta-glucosaminy1)aspartagine amidas) (18) which was purchased from Genzyme (Boston, MA). The glycopeptides from 5 mg of NaBH₄ dissolved in 200 μl of 0.1 M NaOH followed by 3 mg of NaBH₄ dissolved in 200 pl of 0.01 M NaOH followed by 3 mg of NaBH₄ dissolved in 200 μl of 0.1 M sodium citrate buffer, pH 4.3, at 37 °C for 24 h. After incubation, the incubation mixture was heated in a boiling water bath for 2 min. The sample was then incubated with 100 milliunits of beef kidney β-N-acetylglucosaminidase in 140 μl of 0.1 M sodium citrate buffer, pH 4.3. In order to inhibit a possible contaminating activity of β-galactosidase, 100 mM (final concentration) galactose was added to this incubation mixture. After further incubation at 37 °C for 24 h, the mixture was heated in a boiling water bath for 2 min. The digestes were then purified by Sephadex G-50 gel filtration followed by HPLC employing a Lichrosorb-NH₂ column as described above. For extensive digestion by a mixture of β-galactosidase and β-N-acetylglucosaminidase, the saccharides were first incubated with C. lampas β-galactosidase and then with beef kidney β-N-acetylglucosaminidase without heat inactivation of β-
galactosidase or the addition of galactose. After total incubation at 37 °C for 48 h, enzymes were inactivated by heating in a boiling water bath for 2 min, and digested saccharides were purified by anion-exchange HPLC. β-Galactosidase from C. lampas and β-N-acetylglucosaminidase from beef kidney were purchased from Sigma and Boehringer Mannheim, respectively.

Determination of Carbohydrate Composition—Sialic acid content was determined by the periodate-resorcinol reaction (25). Neutral sugars and hexosamines were determined after methanolysis in 0.5 N hydrochloric acid in anhydrous methanol at 80 °C for 4 h. Insolstitial was added as an internal standard. After methanalysis, the products were dried under nitrogen and further in vacuo under P₂O₅ and NaOH. The dried products were trimethylsilylated with Tri-Sil (Pierce Chemical Co.). The trimethylsilylated derivatives were then analyzed by gas-liquid chromatograph-mass spectrometry as described.² In parallel, fetuin was analyzed in order to obtain response factors.

RESULTS

Isolation of N-Linked Saccharides from Recombinant Erythropoietin—Erythropoietin was purified from the spent medium of Chinese hamster ovary cells transfected with a human erythropoietin gene and from human urine of aplastic anemic patients as described under "Experimental Procedures." The purified proteins showed a major band with M, 38,000 and a faint band with M, ~80,000 (Fig. 1). The latter band is probably the dimer of erythropoietin. The carbohydrate composition of this molecule is shown in Table I. Glycopeptides were prepared from 5 mg of recombinant erythropoietin by Pronase digestion and isolated by Sephadex G-15 column gel filtration. Glycopeptides (1.5 mg) were then treated with alkaline borohydride to release N-linked oligosaccharides, and the alkaline borohydride-treated samples were applied to a column of Bio-Gel P-4. As shown in Fig. 24, three peaks were detected in addition to a salt peak. The second (fractions 30-35) and the third (fractions 36-40) peaks were found to

²M. Fukuda, M. Lauffenburger, H. Sasaki, E. M. E. Rogers, and A. Dell (1987) J. Biol. Chem., in press.
contain O-linked oligosaccharides (see Miniprint), whereas glycopeptides containing N-linked saccharides eluted at fractions 25–29. Fractions 25–29 were pooled and digested with N-glycanase. The digest, after reduction with NaB3H4, was subjected to Sephadex, carbohydrate structures of N-linked saccharides, the saccharides from Recombinant Erythropoietin—in order to determine the carbohydrate structures of N-linked saccharides, the saccharides were subjected to FAB-MS and methylation analysis. As shown in Fig. 3A, FAB-MS of permethylated N-linked saccharides provided fragment ions at m/z 376 (and 344), 825, 1274, and 1723, which correspond to NeuNAc+, NeuNAc→Hex→HexNAc+, NeuNAc→Hex→HexNAc→Hex→HexNAc+ and NeuNAc→Hex→HexNAc→Hex→HexNAc→Hex→HexNAc+. These results indicate that N-linked saccharides contain N-acetyllactosaminyl repeats in the side chains. This conclusion was supported by the detection of a fragment ion at m/z 1362 for Hex3HexNAc7 when desialylated and methylated saccharides were subjected to FAB-MS (Fig. 4A). Methylation analysis of N-linked saccharides indicates the following features. 1) Galactose is terminal or 3-substituted; 2) all of the N-acetylgalcosamine residues except the reducing terminal residue are substituted at C-4; 3) 2-substituted mannose (0.16 mol), 2,4-substituted mannose (0.78 mol), 2,6-substituted mannose (1.05 mol), and 3,6-substituted mannose (1.0 mol) were detected (Table II). After desialylation, a majority of 3-substituted galactose residues were converted to terminal galactose, indicating that sialic acid is linked to galactose through an α2→3-linkage. However, 0.82 mol of 3-substituted galactose, which corresponds to 17% of the total galactose derivatives, was still detected after desialylation. This amount of galactose is presumably derived from N-acetyllactosaminyl repeats. The same analysis also showed that 85% of reducing terminal N-acetylgalcosamine is substituted with fucose at C-6, whereas the rest of the reducing terminal N-acetylgalcosamine contains no fucose.

These results suggest that the N-linked saccharides of recombinant erythropoietin are mainly composed of tetraantennary saccharides with or without N-acetyllactosaminyl repeats.

Isolation of Asialo N-Linked Saccharides—N-Linked saccharides were subjected to further analysis. The asialo form of IgG saccharide from fetuin (arrowhead 1), the trisialosyl saccharide from fetuin (arrowhead 3), and the asialo form of IgG saccharide (arrowhead 2).

TABLE I
Carbohydrate composition of erythropoietin

|          | Urinary erythropoietin | Recombinant erythropoietin |
|----------|------------------------|-----------------------------|
| Fuc      | 2.9                    | 4.1                         |
| Man      | 9.2                    | 8.7                         |
| Gal      | 12.9                   | 13.8                        |
| GlcNAc   | 16.3                   | 17.2                        |
| GalNAc   | 0.9                    | 0.9                         |
| NeuNAc   | 10.4                   | 9.5                         |

Numbers are expressed as moles/mole of erythropoietin.
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Fig. 3. Fast atom bombardment-mass spectra of permethylated total N-linked saccharides from recombinant erythropoietin (A) and urinary erythropoietin (B). The positive spectra were recorded. A, fragment ions were detected at m/z 376 (and 344) for NeuNAc′, 580 for NeuNAc′-Hex′, 825 (and 793) for NeuNAc′-Hex-NeuNAc′, 1274 for NeuNAc′-Hex-NeuNAc′-Hex, and 1723 for NeuNAc′-Hex-NeuNAc′-Hex-NeuNAc′. A small peak was detected also at m/z 464 for Hex-NeuNAc′. B, in addition to the ions described above, a minor fragment ion was detected at m/z 913 for Hex-NeuNAc′-Hex-NeuNAc′.

Structures of Biantennary Saccharides and Triantennary Saccharides—The elution position of the biantennary saccharides (peak 2) was identical to the IgG saccharide which is mainly composed of Galβ1-GlcNAcβ1-Manα1-GlcNAcβ1-Fuc. In addition, this saccharide bound to a concanavalin A-Sepharose and was eluted by 20 mM methyl-α-glucoside. Those results establish that this fraction (peak 2) is a typical complex saccharide with biantennary side chains.

Methylation analysis (Table II) of the triantennary saccharides (peak 3) provided 0.65 mol of 2,6-substituted mannose (3,4-di-O-methylmannose) and 0.35 mol of 2,4-substituted mannose (3,6-di-O-methylmannose) in addition to 1 mol each of 2- and 3,6-substituted mannose (Table II). The saccharides were also digested by a mixture of β-galactosidase and β-N-acetylgalactosaminidase to yield Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4(Fluco1→6)GlcNAcOH, as judged by HPLC with a Lichrosorb-NH2 column followed by methylation analysis. These results established the structures of the triantennary saccharides, as shown in Table III.

In order to elucidate which of the outer α-mannosyl residues is disubstituted at C-2 and C-4, the N-linked saccharides (fractions 29–41 in Fig. 2B) were subjected to periodate oxida-
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Fig. 5. HPLC of asialo N-linked saccharides derived from erythropoietin. N-Linked saccharides were desialylated by mild acid hydrolysis, applied to a 5-μm Lichrosorb-NH₂ column, as described under "Experimental Procedures." The effluent was monitored by measuring the absorbance at 202 nm. A and B, total N-linked saccharides from recombinant erythropoietin (A) and urinary erythropoietin (B); C–E, N-linked saccharides from recombinant erythropoietin separated by TSK-DEAE ion-exchange chromatography as shown in Fig. 7A. Monosialosyl (C), disialosyl (D), and trisialosyl (E) fractions were desialylated, applied to the Lichrosorb-NH₂ column, and eluted under the same conditions. Ordinate, relative intensity at A₀₀₀ nm; abscissa, retention time.
| Saccharide | Structure | Relative amount |
|------------|-----------|-----------------|
| Biantennary | Galβ1→4GlcNAcβ1→2Manα1 | 1.4% |
| Triantennary | Galβ1→4GlcNAcβ1 | 3.5% |
| Triantennary Lac1 | Galβ1→4GlcNAcβ1 | 6.5% |
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| Seccharide | Structure | Relative amount % |
|---|---|---|
| Tetraantennary Lac₁ | Galβ₁→4GlcNAcβ₁ | 3.2 |
| | Galβ₁→4GlcNAcβ₁→3Galβ₁→4GlcNAcβ₁ | 6 |
| | Galβ₁→4GlcNAcβ₁ | 3 |
| | Manα₁ | Fuc α₁ | 6 |
| | Galβ₁→4GlcNAcβ₁ | 2 |
| Tetraantennary Lac₂ | Galβ₁→4GlcNAcβ₁→3Galβ₁→4GlcNAcβ₁ | 13.2 |
| | Galβ₁→4GlcNAcβ₁ | 3 |
| | Manα₁ | Fuc α₁ | 6 |
| | Galβ₁→4GlcNAcβ₁ | 2 |
| Tetraantennary Lac₂ | Galβ₁→4GlcNAcβ₁→3Galβ₁→4GlcNAcβ₁ | 3.3 |
| | Galβ₁→4GlcNAcβ₁ | 3 |
| | Manα₁ | Fuc α₁ | 6 |
| | Galβ₁→4GlcNAcβ₁ | 6 |
| | Galβ₁→4GlcNAcβ₁ | 2 |
| | Galβ₁→4GlcNAcβ₁ | 1 |
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*T 15% of the saccharides lack fucose attached to the reducing terminal N-acetylgalactosamine.*
dation followed by reduction and mild acid hydrolysis (Smith degradation) as described (26). Methylation analysis of the product provided 2,4,6-tri-O-methylmannose with the concomitant loss of 3,4-di-O-methylmannose. These results indicate that the 2,4-disubstituted α-mannose is linked to C-3 of β-mannose, as shown in Table III.

Structure of Asialo Tetraantennary Saccharides and Triantennary Saccharides with One N-Acetyllactosaminyl Repeat—
FAB-MS of the saccharides which eluted at peak 4 provided a molecular ion at m/z 3135 corresponding to (Fucl-Hex-HexNAc)n (Fig. 4B). This ion was associated with A-type ions at m/z 2668 for Hex-HexNAc and at m/z 464 (and 432) for Hex→HexNAc+. In addition, an ion at m/z 913 corresponding to Hex→HexNAc→Hex→HexNAc+ was detected. The latter result suggests that this peak contains a triantennary saccharide with one N-acetyllactosaminyl repeat. The presence of a triantennary saccharide was confirmed by methylation analysis (Table II). A small amount (0.11 mol) of 2-substituted mannose (3,4,6-tri-O-methylmannose) was detected as well as 1 mol of 2,4-substituted and 0.9 mol of 2,6-substituted mannose. This result indicates that a triantennary saccharide with 2,4- and 2-substituted α-mannose is present in this saccharide fraction. This triantennary saccharide presumably contains one N-acetyllactosaminyl repeat. The presence of 3-substituted galactose supports the conclusion that this triantennary saccharide contains one N-acetyllactosaminyl repeat (Table II).

The saccharides were sequentially digested with β-galactosidase and β-N-acetylglucosaminidase and subjected to HPLC. As shown in Fig. 6A, the products provided two peaks: the first peak eluted at 12 min corresponds to Man3, GlcNAc±(±Fuc)GlcNAcOH, and the second peak at 15 min corresponds to Gal-GlcNAc-Man, GlcNAc±(±Fuc)GlcNAcOH. The ratio of two peaks was found to be 1:0.9:19. These results indicate that about 15% of the saccharides are triantennary saccharides with one N-acetyllactosaminyl repeat, whereas 85% of the saccharides are tetraantennary saccharides. These two molecular species, however, will provide the same molecular ion at m/z 3135 on FAB-MS analysis.

In order to elucidate which side chains were elongated to form the N-acetyllactosaminyl repeat, the saccharides which eluted at 15 min in Fig. 6A (denoted as 1) were methylated. The saccharides provided 1 mol of 4-substituted mannose (2,3,6-tri-O-methylmannose), 3,6-substituted mannose (2,4-di-O-methylmannose), terminal mannose (2,3,4,6-tetra-O-methylmannose), terminal galactose (2,3,4,6-tetramethylgalactose), 2 mol of 4-substituted N-acetylglucosamine (3,6-di-O-methyl-N-methylacetylgalactosamine), and 1 mol of 4,6-substituted N-acetylglucosamine (1,3,5,6-tetra-O-methyl-N-acetylgalactosaminitol). These results indicate that the N-acetyllactosaminyl repeat is linked to C-4 of α-mannose, as shown in Table III.

The structure of the saccharide “core” (see Fig. 6A) was confirmed by methylation analysis and FAB-MS to be Man1→6 (Man1→3) Man1→6 GlcNAc1→4 (Fuc1→6) GlcNAcOH (89%) and Man1→6 (Man1→3) Man1→6 GlcNAc1→4 GlcNAcOH (11%). Combining these results with exoglycosidase digestion, the structure of the core was elucidated to be Man1→6 (Man1→3) Man1→6 GlcNAc1→4 GlcNAc1→4 (± Fuc1→6) GlcNAcOH. All of the saccharides including tetraantennary with or without N-acetyllactosaminyl repeats were converted to this core saccharide when they were digested with a mixture of β-galactosidase and β-N-acetylglucosaminidase.

Structure of Asialo Tetraantennary Saccharides with One N-Acetyllactosaminyl Repeat (Lac) — Methyl analysis of peak 5 in Fig. 5A provided 1 mol of 2,4-substituted mannose, 2,6-substituted mannose, and 3,6-substituted mannose (Table II), indicating that peak 5 is composed of tetraantennary saccharides. The presence of 3-substituted galactose (0.8 mol) in this asialo form indicates that the saccharides contain one N-acetyllactosaminyl repeat. FAB-MS of the saccharides after permethylation yielded a signal at m/z 913 for Hex→HexNAc→Hex→HexNAc+ (Fig. 4C), which is indicative of one N-acetyllactosaminyl repeat.

The saccharides of peak 5 were sequentially digested by β-galactosidase and β-N-acetylglucosaminidase and subjected to HPLC. As shown in Fig. 6B, the major product eluted at 15 min, which corresponds to Gal-GlcNAc-Man, GlcNAc±(±Fuc)GlcNAcOH. The methylation analysis of this product provided 0.1 mol of 2-substituted mannose, 0.1 mol of 4-substituted mannose, 0.8 mol of 6-substituted mannose, and 1 mol each of terminal mannose and 3,6-substituted mannose as mannose derivatives. These results indicate that 80% of the N-acetyllactosaminyl repeat is attached to C-6 of 2,6-substituted mannose, 10% is attached to C-4 of 2,4-substituted mannose, and 10% is attached to C-2 of either 2,6- or 2,4-substituted mannose (Table III).

Structure of Tetraantennary Saccharides with Two N-Acetyllactosaminyl Repeats (Lac2) — Methylation analysis of peak 6 in Fig. 5A provided 1.7 mol of 3-substituted galactose, 4 mol of terminal galactose, and 1 mol each of 2,4-substituted, 2,6-substituted, and 3,6-substituted mannose, in addition to other derivatives (Table II). The results indicate that this saccharide fraction consists of tetraantennary saccharides with two N-acetyllactosaminyl repeats. FAB-MS of this oligosaccharide fraction supported the above conclusion since a fragment ion at m/z 913 corresponding to Hex→HexNAc→Hex→HexNAc+ was detected (Fig. 4D).

The saccharides were digested sequentially by β-galactosidase and β-N-acetylglucosaminidase to yield a major peak at 19 min (peak 2 in Fig. 6C), which corresponds to (Gal-GlcNAc)n Man1, GlcNAc±(±Fuc)GlcNAcOH, confirming that the starting sample contains tetraantennary saccharides with two N-acetyllactosaminyl repeats. Methylation analysis of this product provided 1 mol of terminal mannose, 0.8 mol of 2,6-substituted mannose, and 0.2 mol each of 6- and 4-substituted mannose, in addition to 1 mol each of 3,6-substituted mannose, terminal galactose, and reducing terminal N-acetylglucosamine and 2 mol of 4-substituted N-acetylglucosamine. However, 2,4-substituted mannose or 3-substituted galactose was not detected. These results indicate that each side chain arising from 2,6-substituted mannose was elongated by one N-acetyllactosaminyl repeat in 80% of the saccharides. In addition, 20% of the molecules have N-acetyllactosaminyl repeats in side chains elongating from C-6 and C-4 (Table III).

Structure of Tetraantennary Saccharides with Three N-Acetyllactosaminyl Repeats (Lac3) — The saccharides in peak 7 eluted at the position where saccharides containing seven N-acetyllactosaminyl units are expected to elute. This is because the difference of the elution time between peaks 7 and 6 is the same as that between peaks 6 and 5. The saccharides were sequentially digested with β-galactosidase and β-N-acetylglucosaminidase, and the products were analyzed by HPLC. As shown in Fig. 6D, a major peak eluted at 24 min, which corresponds to (Gal-GlcNAc)n Man1, GlcNAc±(±Fuc)GlcNAcOH. However, about 35% of the products eluted at the position corresponding to (Gal-GlcNAc)n Man1, GlcNAc±(±Fuc)GlcNAcOH. These results suggest that 65% of the peak 7 saccharides are tetraantennary saccharides with three N-acetyllactosaminyl repeats (Lac3), whereas 35% of the saccharides are
tetraantennary saccharides with two N-acetyllactosaminyl repeats (Lac$_3$). These results were confirmed by methylation analysis, as shown in Table II. Methylation analysis of the saccharides provided 2.4 mol of 3-substituted galactose, 4 mol of terminal galactose, and 1 mol each of 2,4-, 2,6-, and 3,6-substituted mannose. The same analysis indicates that about 45% of the reducing terminal N-acetylglucosamine was un-reduced judging from the amount of 3-O-methyl-N-acetylglucosamine. This compound is produced when the reducing terminal N-acetylglucosamine is incompletely reduced before methylation.

In order to elucidate which side chains were elongated to form the N-acetyllactosaminyl repeat, the saccharides which eluted at 24 min (peak 3 in Fig. 6D) were methylated. The saccharides provided 0.6 mol of 2,6-substituted mannose, 0.4 mol of 6-substituted mannose, 0.3 mol of 4-substituted mannose, 0.3 mol of terminal mannose, 0.4 mol of 2-substituted mannose, and 1 mol of 3,6-substituted mannose as mannose derivatives. These results indicate that each side chain arising from 2,6-substituted mannose and the side chains arising from C-2 or C-4 of 2,4-substituted mannose were elongated by N-acetyllactosaminyl units.

Since FAB-MS of the starting materials afforded a fragment ion at m/e 1723 for NeuNAc→Hex→HexNAc→Hex→HexNAc→Hex→HexNAc (see Fig. 5A), it is likely that Lac$_3$ saccharides contain three N-acetyllactosaminyl units in one of the side chains which are attached to C-2 or C-6 of 2,6-substituted mannose or C-4 of 2,4-substituted mannose. This was confirmed by the fact that peak 3 in Fig. 6D provided a small amount (0.1 mol) of 3-substituted galactose on methylation analysis.

Structures of Asialo N-Linked Saccharides from Recombinant Erythropoietin—The results obtained above are summarized in Table III. By measuring radioactivity in each fraction, the relative yields of saccharides were calculated. In some cases, it was necessary to obtain the ratio after exoglycosidase digestion. For example, the ratio of triantennary with one N-acetyllactosaminyl repeat and tetraantennary in peak 4 was obtained in Fig. 6A.

Fractionation of Intact N-Linked Saccharides by TSK-DEAE Ion-exchange Chromatography—in order to determine how these saccharides are sialylated, intact N-linked saccharides were fractionated by HPLC employing a TSK-DEAE column. As shown in Fig. 7A, sialylated saccharides were essentially separated into four fractions: monosialosyl (fraction I) (70% of the total saccharide), disialosyl (fraction II) (41%), trisialosyl (fraction III) (48%), and tetrasialosyl (fraction IV) (4%) saccharides. No detectable amount of carbohydrates was present in other fractions.

After desialylation, monosialosyl saccharides (fraction I) were found to contain biantennary (12% of the total monosialosyl saccharides), triantennary (17%), tetraantennary (plus triantennary with one N-acetyllactosamine repeat (28%), tetraantennary with one N-acetyllactosamine repeat (29%), tetraantennary with two N-acetyllactosamine repeats (11%), and tetraantennary with three N-acetyllactosamine repeats (3%) (Fig. 5C). Disialosyl saccharides (fraction II) were found to consist of triantennary (70% of the total disialosyl saccharides), tetraantennary (plus triantennary with one N-acetyllactosamine repeat (43%), and tetraantennary saccharides with one (36%), two (12%), or three (2%) N-acetyllactosamine repeats (Fig. 5D). Trisialosyl saccharides (fraction III), however, contain only tetraantennary (47% of the total tetrasialylated saccharides) and tetraantennary saccharides with one (39%), two (12%), or three (2%) N-acetyllactosaminyl repeats (Fig. 5E). The amount of tetrasialylated saccharides was significantly low (4.2% of the total). Those saccharides were found to contain tetraantennary saccharides and tetraantennary saccharides with one or two N-acetyllactosaminyl repeats (data not shown).

These results indicate that 1) the biantennary saccharide is almost exclusively in a monosialylated form; 2) the triantennary saccharides are in monosialylated or disialylated forms; 3) the tetraantennary saccharides are mostly in disialylated or trisialylated forms; and 4) the tetraantennary saccharides with one, two, or three N-acetyllactosaminyl repeats are mostly in disialylated or trisialylated forms. These results suggest that one of the side chains in the saccharides is almost always terminated without a sialic acid residue (see below).

In order to know whether any difference exists among different batches of recombinant erythropoietin, two additional batches (Batches 3 and 4 in Table I) of recombinant erythropoietin were subjected to analysis. Interestingly, these samples contained more highly sialylated saccharides: the disialosyl form is 18-21% of the total saccharides; the trisialosyl form is 64-67%; the tetrasialosyl form is 10-13%; and the monosialosyl form is less than 6%. However, the relative ratios of asialo biantennary, triantennary, tetraantennary, and tetraantennary saccharides with one, two, or three N-acetyllactosaminyl repeats were almost identical among different samples. These results indicate that sialylation may vary among different batches of recombinant erythropoietin but their backbone structures are the same.

Separation of Saccharides with Different Backbone Structure but with the Same Number of Sialic Residues in Side Chains—The results of Fig. 7A suggested to us that each peak in the monosialosyl, disialosyl, or trisialosyl fraction may represent saccharides with different backbone structures but with the same number of sialic acid residues. In order to test this possibility, another 5 mg of recombinant erythropoietin (Batch 2 in Table I) was treated to yield N-linked saccharides, and these saccharides were fractionated by TSK-DEAE ion-exchange chromatography. This sample provided an elution profile almost identical to that in Fig. 7A. Saccharides were divided into four (fraction II) or three (fraction III) fractions, desialylated, and subjected to another HPLC employing a Lichrosorb column. Fraction II-1, which eluted earliest in TSK-DEAE chromatography, provided tetraantennary saccharides with two or three N-acetyllactosaminyl repeats (Fig. 8A, see Miniprint), whereas the last peak (fraction II-4) mainly consists of triantennary and tetraantennary saccharides (Fig. 8D). Similarly, the earliest peak in the trisialosyl fraction (fraction III-1) provided Lac$_3$ and a small amount of Lac$_2$ and Lac$_0$ (Fig. 8E), whereas the last peak (fraction III-3) provided almost exclusively tetraantennary saccharides (Fig. 8E). These results indicate that the saccharides with higher numbers of N-acetyllactosaminyl units elute earlier than those with smaller numbers of N-acetyllactosaminyl units in TSK-DEAE ion-exchange chromatography.

Localization of α2→3-Linked Sialic Acid in the Side Chains—in order to know which side chains are preferentially sialylated, fractions II-2, II-3 and III-3 were digested extensively with a mixture of β-galactosidase and β-N-acetylglucosaminidase, and the products were purified by Sephadex G-50 gel filtration followed by TSK-DEAE chromatography. The purified products were then subjected to methylation analysis, and the results are summarized as follows.

Disialosyl Tetraantennary Saccharides with One or Two N-acetyllactosaminyl Repeats (Fraction II-2)—Methylation analysis on the exoglycosidase product of fraction II-2 provided the following mannose derivatives: 0.9 mol each of 2,6-
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Fig. 7. Ion-exchange HPLC of N-linked saccharides obtained from recombinant erythropoietin (A) and urinary erythropoietin (B). N-Linked oligosaccharide fractions obtained after Sephadex G-50 gel filtration (fractions 29-41 in Fig. 2B) were applied to a column of TSK-DEAE SW2 equilibrated with 25 mM potassium phosphate buffer, pH 5.0. After washing with the same buffer for 10 min, the column was eluted with a linear gradient from the same buffer to 400 mM potassium phosphate buffer, pH 5.0. A portion of the fractions indicated by the horizontal arrows were pooled and subjected to HPLC with a Lichrosorb column, as shown in Figs. 5 (C-E) and 8.

Fig. 11. Proposed structures of tetraantennary saccharides (A) and tetraantennary saccharides with N-acetyllactosaminyl repeats (B) obtained from recombinant erythropoietin. A, sialylation takes place preferentially at the side chain arising from C-6 and then from the side chain at C-2 of 2,6-substituted mannose and C-4 of 2,4-substituted mannose. B, the saccharides with one N-acetyllactosaminyl repeat \( (m = 1, n = 0, o = 0) \), in 90% of the molecules; and the saccharides with two N-acetyllactosaminyl repeats \( (m = n = 1, o = 0) \), in 10% of the molecules; and the saccharides with three N-acetyllactosaminyl repeats \( (m + n + o = 3) \).

substituted and terminal mannose, 0.1 mol of 4- and 6-substituted mannose, and 1 mol of 3,6-substituted mannose. No other mannose derivatives, including 2,4-substituted mannose, were detected. The results indicate that the 2 sialosyl residues are almost exclusively linked to the side chains arising from C-2 and C-6 of 2,6-substituted mannose and that from C-2 of 2,4-substituted mannose.

Disialosyl Tetraantennary Saccharide (Fraction II-3)—

Trisialosyl Tetraantennary Saccharide (Fraction III-3)—

Methylation analysis after exoglycosidase digestion of fraction III-3 provided the following mannose derivatives: 1 mol each of 2,6- and 3,6-substituted mannose and 0.5 mol each of 2,4- and 4-substituted mannose. However, no other mannose derivative was detected. The results indicate that the nonsialylated chain almost exclusively arises from C-2 of 2,4-substituted \( \alpha \)-mannose.
Structure of Sialylated Tetraantennary Saccharides with or without N-Acetyllactosamine Repeats—Based on the results described above, the structure of intact tetraantennary saccharides and those with N-acetyllactosaminyl repeats, which represent 85% of the total saccharides, can be proposed as shown in Fig. 11. The tetraantennary saccharides are mainly present as disialosyl or trisialosyl forms, and 2-3-linked sialic acid is attached to the side chains arising from C-6 and C-2 of 2,6-substituted mannose and C-4 of 2,4-substituted mannose. In the tetraantennary saccharides with N-acetyllactosaminyl repeats, sialic acid residues are always present in the side chain which contains N-acetyllactosaminyl repeats. This conclusion was supported by FAB-MS analysis. As shown in Fig. 3A, all of the fragment ions containing polylactosaminyl units are sialylated.

In order to delineate this further, trisialylated saccharides (fraction III) were extensively digested by β-galactosidase and β-N-acetylglucosaminidase. The methylation analysis of this product showed that more than 90% of the side chain attached to C-2 of 2,4-substituted mannose was terminated without sialic acid. These combined results support the proposed structures shown in Fig. 11.

Structure of Carbohydrate Units of Urinary Erythropoietin—Since only a limited amount of urinary erythropoietin was available, the following experiments were carried out to analyze carbohydrate units of urinary erythropoietin. Glycopeptides, prepared by Pronase digestion of urinary erythropoietin, were subjected to alkaline borohydride treatment. The alkaline borohydride-treated sample was then applied to Bio-Gel P-4 gel filtration. Urinary erythropoietin saccharides provided almost the same elution profile as Fig. 2A. The glycopeptides containing N-linked saccharides (fractions 24–29) were digested by N-glycanase, and the digest was subjected to Sephadex G-50 gel filtration. Again, the elution profile of N-linked saccharides from urinary erythropoietin was almost identical to that from recombinant erythropoietin (see Fig. 2B).

Methylation analysis of N-linked saccharides (fractions 23–41 after Sephadex G-50 gel filtration) provided partially O-methylated monosaccharide derivatives, which are almost identical to those produced from highly sialylated Batch 3 of recombinant erythropoietin (Table II). FAB-MS of per-methylated N-linked saccharides provided fragment ions for NeuNAc* (m/z 376 and 344), NeuNac→Hex→HexNAc* (m/z 825), NeuNac→Hex→HexNAc→Hex→HexNAc* (m/z 1274), and NeuNac→Hex→HexNAc→Hex→HexNAc→Hex→HexNAc* (m/z 1723) (Fig. 3B). These results are essentially the same as those obtained on recombinant erythropoietin (compare Fig. 3, A and B).

N-Linked saccharides were then desialylated and subjected to HPLC employing a Lichrosorb-NH$_2$ column. As shown in Fig. 5B, urinary erythropoietin saccharides provided triantenary, tetraantennary, and tetraantennary saccharides with one, two, or three N-acetyllactosaminyl units. The relative proportion among these saccharides is almost identical to that obtained on recombinant erythropoietin except that urinary erythropoietin apparently lacks biantennary saccharides. In order to determine the relative amounts of sialylated N-linked saccharides, intact N-linked saccharides were subjected to TSK-DEAE ion-exchange chromatography. Fig. 7B shows that N-linked saccharides from urinary erythropoietin contain disialosyl (27% of the total saccharides), trisialosyl (56%), and tetrasialosyl (17%) saccharides. These results indicate that urinary erythropoietin and recombinant erythropoietin have an almost identical set of N-linked saccharide units but with slightly different sialylation depending upon the batches of recombinant erythropoietin (see above and Table I).

DISCUSSION

This paper reports the detailed structures of the carbohydrate moiety of human erythropoietin produced by recombinant DNA. The protein analyzed was produced in Chinese hamster ovary cells which were transfected with human erythropoietin cDNA (7). As far as we are aware, this is the first report on the detailed carbohydrate structure of a glycoprotein produced by recombinant DNA in comparison with the glycoprotein of natural origin. Although Mutsaers et al. (28) reported the carbohydrate structure of human γ-interferon produced in Chinese hamster ovary cells, their studies did not investigate those of naturally occurring human γ-interferon. The carbohydrate composition (Table I) showed that erythropoietin contains three N-linked saccharides and one O-linked saccharide, and these conclusions are consistent with the recent report on the amino acid sequence of human urinary erythropoietin (29).

The present study revealed that a large proportion of the carbohydrate moiety of recombinant erythropoietin is composed of tetraantennary saccharides with one (32.1% of the total saccharides), two (16.5%), and three (4.7%) N-acetyllactosaminyl repeats. The localization of these polylactosaminyl units was elucidated by sequential exoglycosidase digestion followed by methylation analysis, and the results are summarized as follows (see also Table III).

When the saccharides contain one N-acetyllactosaminyl repeat, more than 70% of this repeat is preferentially attached to the side chain arising from C-6 of 2,6-substituted mannose, and 19% of the repeat is attached to that from C-4 of 2,4-substituted mannose. When the saccharides contain two N-acetyllactosaminyl repeats, these repeats are attached to C-2 and C-6 of 2,6-substituted mannose in 80% of the molecules. The rest of the molecule contains N-acetyllactosaminyl repeats in the side chains arising from C-6 of 2,6-substituted mannose and C-4 of 2,4-substituted mannose. These results indicate that N-acetyllactosaminyl repeats are most preferentially added to C-6 of 2,6-substituted mannose and then to C-2 of 2,6-substituted mannose. These conclusions are consistent with previous reports on several cellular glycoproteins. For example, Cummings and Kornfeld (30) reported that the mouse lymphoma BW5147 cell line expressed a significant amount of polylactosaminoglycan, whereas its mutant, which lacks the side chain arising from C-6 of 2,6-substituted mannose, expresses a minimum amount of polylactosaminoglycan. Li et al. (31) isolated polylactosaminoglycan from Chinese hamster ovary cells in which polylactosaminyl units are attached to C-2 and C-6 of 2,6-substituted mannose. Similarly, polylactosaminyl units were found in triantenary and tetraantennary saccharides of various origins (24, 32–34). These results appear to establish that N-acetyllactosaminyl repeats are preferentially added to C-6 of 2,6-substituted mannose and then to C-2 of 2,6-substituted mannose. These 2,6-substituted mannose residues are usually linked to the C-6 side of β-mannose. In human erythrocytes, polylactosaminyl elongation can be found in the side chain arising from C-2 of α-mannose which is linked to C-6 of β-mannose (23, 35). It is likely that human erythroid cells contain very little activity of the N-acetylgalactosaminyltransferase which forms a GlcNAcβ1→6Man branch. As a result, N-acetyllactosamine repeats are formed on the secondary preferable side chain, which is attached to C-2 of α-mannose linked to C-6 of β-mannose, in these erythroid cells.

This study showed that human erythropoietin exclusively
contains α2→3-linked sialic acid. This fact allowed us to elucidate the localization of 2→3-linked sialic acid residues among different side chains. This was achieved by extensive digestion of intact saccharides with β-galactosidase and β-N-acetylgalactosaminidase followed by methylation analysis of the products. These results can be summarized as follows. 1) When typical triantennary or tetraantennary saccharides contain 2 sialic acid residues, they are attached to C-2 and C-6 of 2,6-substituted mannose or C-6 of 2,6-substituted mannose and C-4 of 2,4-substituted mannose. 2) When saccharides with N-acetyllactosaminyl repeats contain 2 sialic acid residues, they are attached almost exclusively to C-2 and C-6 of 2,6-substituted mannose. This localization is essentially identical to that of N-acetyllactosamine repeats. Thus, it is apparent that polylactosamine is preferably sialylated through an α2→6-linkage.

These results are consistent with our previous results obtained on polylactosaminoglycans from chronic myelogenous leukemia cells; α2→3-linked sialic acid is present on side chains arising from C-6 and C-2 of 2,6-substituted mannose and C-4 of 2,4-substituted mannose, and those side chains are longer than that terminating with 2→6-linked sialic acid (24). Similar results were obtained in human erythrocyte Band 3 polylactosaminoglycans; polylactosaminyl side chains arising from the C-6 side of β-mannose are sialylated through a 2→3-linkage, whereas the shorter chain arising from the C-3 side is sialylated through a 2→6-linkage (23, 35). Similarly, Yamashita et al. (33) and Markle and Cummings (36) found that longer polylactosaminy1 side chains are almost exclusively sialylated through a 2→3-linkage. Interestingly, short polylactosamine chains in thyroid cell glycoprotein Glp-1 (37) and BW5147 (36) are terminated with 2→6-linked sialic acid. Our results also showed that almost no 2→3-linked sialic acid is attached to the side chain arising from C-2 of 2,4-substituted mannose. It is noteworthy that this side chain was found to be exclusively sialylated through a 2→6-linkage in many glycoproteins including normal and leukemic granulocyte polylactosaminoglycans (19-21, 24, 34, 38-41).

By using a bovine colostrum α2→6-sialyltransferase, Joziassi et al. (42) have shown that preferential sialylation takes place first on C-2 of 2,4-substituted mannose and then on C-4 of 2,4-substituted mannose. This branch (or side chain) specificity appears to be opposite to the distribution of 2→3-linked sialic acid. Thus, it is likely that α2→6-sialyltransferase and α2→4-sialyltransferase have complementary specificity toward different side chains. Furthermore, our results raise the possibility that the side chains containing polylactosaminy1 units would be preferable sites for 2→5-linked sialylation.

This study demonstrated that the carbohydrate moiety of human erythropoietin isolated from human urine is indistinguishable from that of recombinant erythropoietin except for a difference in degree of sialylation. Urinary erythropoietin has a similar degree of sialylation as the highly sialylated batch of recombinant erythropoietin (Tables I and II). The recombinant erythropoietin was produced in Chinese hamster ovary cells, and urinary erythropoietin is presumably derived from human kidney cells. The results therefore suggest two possibilities. 1) Chinese hamster ovary and human kidney cells contain similar glycosyltransferases. 2) The protein acceptor itself influences glycosylation even when a similar set of glycosyltransferases are not present in two cell types. It will be interesting to see if the carbohydrate moiety of erythropoietin produced in other mammalian cells is similar to those elucidated in this study. This study also demonstrated that the major carbohydrate units of erythropoietin are tetraantennary saccharides with or without N-acetyllactosamine repeats. It has been shown that rat liver cells uptake the asialo form of glycoproteins which contain tri- or tetraantennary saccharides (43). It is therefore reasonable that the asialo form of erythropoietin is taken up by liver cells through a galactose-binding protein (15). Our preliminary studies showed that a portion of intact erythropoietin of both recombinant and urinary origins is taken up by rat liver cells, presumably because of the incomplete sialylation. It will be interesting to test if sialylation by α2→6-sialyltransferase elongates the serum concentration of erythropoietin and sustains in vivo activity longer than the starting erythropoietin.

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REFERENCES
1. Goldwasser, E., and Kung, C. K. H. (1968) Ann. N. Y. Acad. Sci. 149, 49-53
2. Jacobson, L. O., Goldwasser, E., Fried, W., and Pilak, L. F. (1957) Nature 179, 635-636
3. Fried, W. (1972) Blood 40, 671-677
4. Zanjani, E. D., Asensao, J. L., McGlave, P. B., Banisadre, M., and Ash, R. C. (1981) J. Clin. Invest. 67, 1183-1188
5. Adamson, J. W., Eschbach, J. W., and Finch, C. A. (1988) Am. J. Med. 44, 725-733
6. Blose, T., Huang, C. F.-H., and Goldwasser, E. (1977) J. Biol. Chem. 252, 5558-5564
7. Jacobs, K., Shoemaker, C., Ruslersdorff, R., Neill, S. D., Kaufman, R. M., Mufson, A., Seehra, J., Jones, S. S., Hewick, R., Fitch, E. F., Kawakita, M., Shimizu, T., and Miyake, T. (1985) Nature 313, 806-810
8. Lim, F. K., Suggs, S., Lin, C. H., Browne, J. K., Smalls, E., Egie, J. C., Chen, K. D., Fox, G. M., Martin, F., Stabinsky, Z., Badrawi, S. M., Lai, M., and Goldwasser, E. (1985) Proc. Natl. Acad. Sci. U. S. A. 83, 7580-7584
9. Powell, J. S., Berkner, K. L., Lebo, R. V., and Adamson, J. W. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 6465-6469
10. Winearls, C. G., Oliver, D., O’Pippard, M. J., Reid, C., Downing, M. R., and Cotes, P. M. (1986) Lancet ii, 1175-1178
11. Eschbach, J. W., Egie, J. C., Dowming, M. R., Browne, J. K., and Adamson, J. W. (1987) N. Engl. J. Med. 316, 73-78
12. Ramachand, R. A., Sawai, R. A., Cooper, J. A. D., and APT, H. L. (1958) Proc. Soc. Exp. Biol. Med. 99, 482-483
13. Lowy, P. H., Keighley, G., and Borsook, H. (1960) Nature 185, 102-103
14. Lukowsky, W. A., and Painter, R. H. (1972) Can. J. Biochem. 50, 999-917
15. Goldwasser, E., Kung, C. K.-H., and Eliason, J. (1974) J. Biol. Chem. 249, 4202-4206
16. Simmonsson, C. C., and Levinson, A. D. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 2495-2499
17. Fukuda, M., Carlsen, S. R., Klock, J. C., and Dell, A. (1986) J. Biol. Chem. 261, 12786-12806
18. Plummer, T. H., Jr., Elder, J. H., Alexander, S., Phelan, A. W., and Tarentino, A. L. (1984) J. Biol. Chem. 259, 10700-10704
19. Nilsson, B., Nordén, N. E., and Svensson, S. (1979) J. Biol. Chem. 254, 4545-4553
20. Biezuner, J. U., and Fiete, D. (1979) J. Biol. Chem. 254, 789-795
21. Endo, M., Suzuki, K., Schmid, K., Fournet, B., Karamanos, Y., Montreuil, J., Dorland, L., Van Halbeek, H., and Vliegenthart, J. F. G. (1982) J. Biol. Chem. 257, 8755-8760
22. Yoshimura, H., Matsumoto, A., Mizuno, T., Kawasumi, T., and Kobata, A. (1981) J. Biol. Chem. 256, 8476-8484
23. Fukuda, M., Dell, A., and Fukuda, M. N. (1984) J. Biol. Chem. 259, 4782-4791
24. Fukuda, M., Bothner, B., Ramsamooj, P., Dell, A., Tiller, P. R., Verci, A., and Klock, J. C. (1985) J. Biol. Chem. 260, 12967-12967
25. Jourdian, G. W., Dean, L., and Roseman, S. (1971) J. Biol. Chem. 246, 430-435
26. Greenwood, F. C., Hunter, W. M., and Glove, J. S. (1963) Biochem. J. 89, 114-123
SUPPLEMENTAL MATERIAL

CARBOHYDRATE STRUCTURE OF ERYTHROPOIETIN EXPRESSED IN CHINESE HAMSTER OVARY CELLS BY A HUMAN ERYTHROPOIETIN CONA

By Hiroshi Saraki, Brian Boymer, Anne Igel and Minoru Fukuda

Isolation of O-linked oligosaccharides from recombinant erythropoietin - The O-linked oligosaccharides were purified from the conditioned medium of HMG-6 cell line expressing the 
erythropoietin gene as described previously (1, 2). The conditioned medium was subjected to TSK-GEL DEAE ion exchange chromatography as shown in Fig. 2.

30-40 were recovered after TSK-DEAE ion exchange chromatography. This fraction contained disialosyl oligosaccharides and tetrasialosyl oligosaccharides. These results, together with the electrophoretic analysis shown in Fig. 3, indicated that the disialosyl oligosaccharide was mono-0-methylated and that the tetrasialosyl oligosaccharide was mono-0-methylated and di-0-methylated. The tetrasialosyl oligosaccharide was tetra-0-methylated. The tri-0-methylated oligosaccharide was sequentially digested by electrophoretic analysis and MALDI-TOF-MS analysis to confirm the identity of the disialosyl oligosaccharide. The tri-0-methylated oligosaccharide was also sequenced by electrophoretic analysis and MALDI-TOF-MS analysis to confirm the identity of the disialosyl oligosaccharide.

30-40 were recovered after TSK-DEAE ion exchange chromatography. This fraction contained disialosyl oligosaccharides and tetrasialosyl oligosaccharides. These results, together with the electrophoretic analysis shown in Fig. 3, indicated that the disialosyl oligosaccharide was mono-0-methylated and that the tetrasialosyl oligosaccharide was mono-0-methylated and di-0-methylated. The tetrasialosyl oligosaccharide was tetra-0-methylated. The tri-0-methylated oligosaccharide was sequentially digested by electrophoretic analysis and MALDI-TOF-MS analysis to confirm the identity of the disialosyl oligosaccharide. The tri-0-methylated oligosaccharide was also sequenced by electrophoretic analysis and MALDI-TOF-MS analysis to confirm the identity of the disialosyl oligosaccharide.

TABLE II. Relative proportions of methylated sugars obtained from O-linked saccharides of recombinant and urinary erythropoietin.

| Sugar            | Recombinant | Urinary 1 | Urinary 2 | Urinary 3 |
|------------------|-------------|-----------|-----------|-----------|
| Methylated Sugar |             |           |           |           |
| 3,4-di-O-methyl  | 1.00        | 1.00      | 1.00      | 1.00      |
| 4-mono-O-methyl  | 0.35        | 0.35      | 0.40      | 0.40      |

TABLE III. Relative proportions of methylated sugars obtained from O-linked saccharides of recombinant and urinary erythropoietin.

| Sugar            | Recombinant | Urinary 1 | Urinary 2 | Urinary 3 |
|------------------|-------------|-----------|-----------|-----------|
| Methylated Sugar |             |           |           |           |
| 3,4-di-O-methyl  | 1.00        | 1.00      | 1.00      | 1.00      |
| 4-mono-O-methyl  | 0.35        | 0.35      | 0.40      | 0.40      |

5 In some cases, the amount of 4-mono-O-methyl was detected in all of the fractions.

6 This was probably because of incomplete reduction.
Carbohydrate Structure of Human Recombinant Erythropoietin

Figure 6. HPLC of asialoforms of N-linked saccharides derived from recombinant human erythropoietin. A. Asialoform saccharides separated by size using a linear gradient of 10% to 45% acetonitrile in 0.1% TFA. B. The digest of saccharide 4. C. The digest of saccharide 5. D. The digest of saccharide 6. E. The digest of saccharide 7.
Carbohydrate Structure of Human Recombinant Erythropoietin

Figure 8. HPLC of each peak obtained by TSK-DEAE ion exchanger chromatography. O-linked oligosaccharides from recombinant erythropoietin were separated by TSK-DEAE ion exchanger chromatography as shown in Fig. 7A. Each peak obtained was desalted and subjected to HPLC with a Lichrosorb-NH2 column at the same conditions of Fig. 5.

- A. 11-1 (fractions 70-77, in Fig. 7A).
- B. 11-2 (fractions 78-81 in Fig. 7A).
- C. 11-3 (fractions 82-87 in Fig. 7A).
- D. 11-4 (fractions 88-91 in Fig. 7A).
- E. 111-1 (fractions 94-98 in Fig. 7A).
- F. 111-2 (fractions 99-102 in Fig. 7A).
- G. 111-3 (fractions 103-108 in Fig. 7A).

Figure 9. Ion-exchange HPLC of O-linked oligosaccharides obtained from recombinant erythropoietin. O-linked oligosaccharides released with alkaline borohydride treatment were isolated by Bio-Gel P-4 gel filtration and those saccharides were separated by TSK-DEAE ion exchange column was equilibrated with 25 mM potassium phosphate buffer, pH 5.0. After washing with the same buffer for 5 min, the column was eluted with a linear gradient from the same buffer to 400 mM potassium phosphate buffer, pH 5.0 over 40 min. The flow rate was 1 ml/min and each fraction contained 0.5 ml. The horizontal arrows indicate the fractions pooled. No carbohydrate was detected in other fractions.

- D-1 (fractions 36-39 in Fig. 7A).
- D-11 (fractions 36-40 in Fig. 7A).
Carbohydrate Structure of Human Recombinant Erythropoietin

Figure 10. Fast atom bombardment-mass spectra of O-linked oligosaccharides from recombinant erythropoietin A, B, C, and D, and purified erythropoietin E. The positive spectra were obtained on permethylated samples.

A: Fractions 18-21 from Fig. 9B. A prominent molecular ion was detected at m/z 873 corresponding to N-linked O-linked HS. These molecular ions are accompanied by fragment ions at m/z 505, 375, and 257 (and 249) for NeuAc, ions at m/z 1062 and 1254 corresponding to a fundamental form of NeuAc, and the mass distribution at m/z 1123, 1114, 1097, and 1080 correspond to an additional NeuAc as NeuAc(2-3)HexNAc. A signal at m/z 555 is probably due to the methylpyruvate of NeuAcHexNAc.

B: Fractions 42-48 from Fig. 9B. A prominent molecular ion was detected at m/z 1224 for NeuAc, NeuAc(2-3)HexNAc. This ion was accompanied by A-type fragment ions at m/z 774, 555, 437, and 329 (86, 344) for NeuAc. The ions at m/z 555 and 86 correspond to NeuAc, and the ions at m/z 329, 437, 555, and 774 probably correspond to a E-oligosaccharide lacking one sialic acid residue.

C: Fractions 41-42 from Fig. 9B. Molecular ions at m/z 1195 and 1193 correspond to NeuAc, NeuAc(2-3)HexNAc, and NeuAc(2-3)HexNAc, respectively. These ions were associated with an ion at m/z 774 and 764 for NeuAc. This fraction also produced fragment ions at m/z 756 and 1194 which are probably derived from unoccupied NeuAc, and A-type fragment ions for NeuAc, NeuAc(2-3)HexNAc. A signal at m/z 1274 corresponds to NeuAc(2-3)HexNAc, and ions probably derived from contaminating E-linked oligosaccharides.

D: O-linked oligosaccharides from purified erythropoietin (fractions 15-21 in Fig. 9B). The A-type fragment ions at m/z 555 and 86 correspond to NeuAc(2-3)HexNAc. The ion at m/z 555 is accompanied by fragment ions at m/z 374 and 344 for NeuAc. A signal at m/z 555 corresponds to NeuAcHexNAc, and ions probably derived from contaminating E-linked oligosaccharides. A cluster of ions 44 mass units apart (marked by crosses) are detectable ions.