EXHIBIT K

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Comparative Study of the Asparagine-linked Sugar Chains of Human Erythropoietins Purified from Urine and the Culture Medium of Recombinant Chinese Hamster Ovary Cells*

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The asparagine-linked sugar chains of human erythropoietin produced by recombinant Chinese hamster ovary cells and naturally occurring human urinary erythropoietin were liberated by hydrazinolysis and fractionated by paper electrophoresis, lectin affinity chromatography, and Bio-Gel P-4 column chromatography. Both erythropoietins had three asparagine-linked sugar chains in one molecule, all of which were acidic complex type. Structural analysis of them revealed that the sugar chains from both erythropoietins are quite similar except for sialyl linkage. All sugar chains of erythropoietin produced by recombinant Chinese hamster ovary cells contain only the NeuAcα2→3Gal linkage, while those of human urinary erythropoietin contain the NeuAcα2→6Gal linkage together with the NeuAcα2→3Gal linkage. The major sugar chains were of fucosylated tetraantennary complex type with and without *N*-acetyllactosamine repeating units in their outer chain moieties in common, and small amounts of 2,4- and 2,6-branched triantennary and biantennary sugar chains were detected. This paper proved, for the first time, that recombinant technique can produce glycoprotein hormone whose carbohydrate structures are common to the major sugar chains of the native one.

Erythropoietin is a glycoprotein hormone involved in the regulation of the level of peripheral erythrocytes (1) by stimulating the differentiation of the erythroid progenitor cells into mature erythrocytes (2). The hormone is primarily produced in the kidney of adults (3). Therefore, the decrease of the erythropoietin production by the destruction of kidney mass (from chronic renal failure (4) or some other reasons) causes anemia. Highly purified erythropoietin is expected to be useful in the therapeutic treatment of such a type of anemia (5). Erythropoietin has been purified from urine of patients with severe aplastic anemia (6), however, it is quite difficult to obtain the sufficient amount of human urinary erythropoietin (urinary HuEPO)[1] for the investigation of its chemical and biological properties and for the clinical application.

In order to overcome this problem, a new approach has been developed by using recombinant DNA techniques. So far, two groups reported the cloning of human erythropoietin gene and its nucleotide sequence analysis (7, 8). Lin *et al.* (8) has succeeded in the expression of the erythropoietin gene in Chinese hamster ovary (CHO) cells by recombinant techniques. The rHuEPO has three possible sites for *N*-glycosylation, Asn-*X*-Ser/Thr (7, 8), and is actually sensitive to *N*-glycanase digestion (9). Recently, Lai *et al.* (43) estimated on the basis of amino acid sequencing data that urinary HuEPO also has three *N*-linked sugar chains and one *O*-linked sugar chain. Analysis of the monosaccharide composition of HuEPO performed in our laboratory confirmed the occurrence of one *N*-acetylgalactosamine residue, indicating that one *O*-linked sugar chain is included in recombinant HuEPO (44). Sugar moiety of urinary HuEPO has been suggested to affect biological properties such as turnover rate, antigenicity, and so on (10–14). Therefore, it is important to elucidate the sugar chain structures of erythropoietin.

Several bioactive glycoproteins have been produced in recombinant mammalian cells (15–17). Among them, the sugar chain structure of human γ-interferon produced in CHO cells has been elucidated; however, its natural counterpart has not been analyzed (17). In this paper, we will describe the comparative analysis of the asparagine-linked sugar chain structures of rHuEPO produced in CHO cells and naturally occurring urinary HuEPO.

EXPERIMENTAL PROCEDURES AND RESULTS[2]

Paper Electrophoresis of Oligosaccharides Released from Erythropoietin by Hydrazinolysis—Radioactive oligosaccharides obtained from rHuEPO and urinary HuEPO by hydrazinolysis were subjected to paper electrophoresis at pH 5.4. As shown in Fig. 1,[3] both samples contained acidic oligosaccharides composed of at least seven components (A-1 to A-7) but no neutral oligosaccharide. When digested with sialidase from *Arthrobacter ureafaciens*, approximately 90% of the total acidic oligosaccharides from rHuEPO and 80% of those from urinary HuEPO were converted to neutral oligosaccharides (data not shown). The residual oligosaccharides migrated

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[1] The abbreviations used are: urinary HuEPO, human erythropoietin purified from the urine of patients with aplastic anemia; CHO, Chinese hamster ovary; rHuEPO, human erythropoietin produced in recombinant CHO cells; ConA, concanavalin A; DSA, *Datura stramonium* agglutinin; AAL, *Aleuria aurantia* lectin; Fuc, fucose.

[2] Portions of this paper (including "Experimental Procedures," part of "Results," and Figs. 3–7) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

[3] Subscript OT is used to indicate $NaB[^3H]_4$-reduced oligosaccharides. All sugars mentioned in this paper were of D-configurations except for fucose, which was of L-configuration.

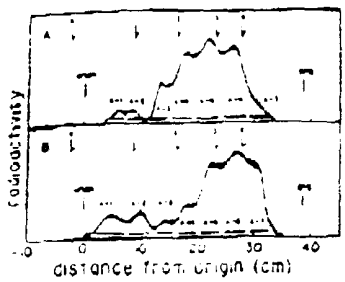

FIG. 1. Paper electrophoresis of the radioactive oligosaccharides obtained from rHuEPO and urinary HuEPO. The oligosaccharides released from rHuEPO (*panel A*) and urinary HuEPO (*panel B*) by hydrazinolysis were subjected to paper electrophoresis at a potential of 73 V/cm for 90 min. The *arrows* indicate the position of authentic oligosaccharides. 0, 1, 2, 3, and 4 indicate non-, mono-, di-, tri-, and tetrasialylated $Gal_3 \cdot GlcNAc_3 \cdot Man_3 \cdot GlcNAc \cdot GlcNAc_{OT}$, respectively

TABLE I

Fractionation of asialo-oligosaccharides released from rHuEPO and urinary HuEPO by serial lectin affinity column chromatography

Fractions*	Molar percent of the total asialo-oligosaccharide fraction	
	rHuEPO	Urinary HuEPO
$ConA^+AAL^+$	4.1	5.3
$ConA^+AAL^-$	1.9	3.7
$ConA^-DSA^+AAL^+$	4.8	2.4
$ConA^-DSA^+AAL^-$	4.1	1.2
$ConA^-DSA^-AAL^+$	76.6	74.6
$ConA^-DSA^-AAL^-$	8.6	12.9

*The symbols represent the bound (+), the passed-through (−), and the retarded (r) fractions, respectively.

around the area corresponding to monosialyl oligosaccharides and were then converted to neutral oligosaccharides by mild methanolysis (0.05 N HCl-methanol, 37 °C, 4 h). From the analysis of each acidic fraction the oligosaccharides, which were partially sensitive to sialidase and susceptible to the methanolysis, were shown to be derived from the small parts of A-3 to A-6 (6–10% of each fraction) and from the major part of A-7 (95% of this fraction) (data not shown). Thus, the result suggested the presence of small amounts of oligosaccharides containing both sialic acid residues and possibly sulfate group. The structures of their neutral portions were common to those of sialidase-sensitive acidic oligosaccharides (data not shown). However, no detailed analysis of them to assign the location of sialic acid and sulfate residues was performed because of the limited amounts of samples.

Fractionation of Asialo-oligosaccharides—The neutral oligosaccharide fractions obtained by sialidase digestion were fractionated by a serial lectin affinity column chromatography. The sample was first applied to a column of ConA-Sepharose. The fraction which passed through the column was then separated into a retarded fraction and a bound fraction by passing through a DSA-Sepharose column. Each of these three fractions, thus obtained, was finally fractionated by a column of AAL-Sepharose. The proportion of each fraction obtained from rHuEPO and urinary HuEPO was summarized in Table I. There were quite similar points between two samples. The first is that the major parts of oligosaccharides were recovered in the $ConA^-DSA^-$ fraction in common: 85.1%

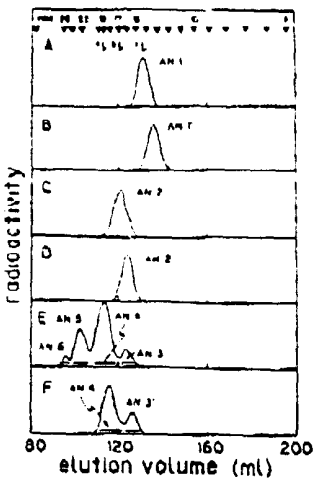

FIG. 2. Bio-Gel P-4 column chromatography of asialo-oligosaccharides. Asialo-oligosaccharides of rHuEPO, fractionated with lectin columns as shown in Table I, were analyzed by Bio-Gel P-4 column chromatography. *Panel A*, the $ConA^+AAL^+$ fraction; *panel B*, the $ConA^+AAL^-$ fraction; *panel C*, the $ConA^-DSA^+AAL^+$ fraction; *panel D*, the $ConA^-DSA^+AAL^-$ fraction; *panel E*, the $ConA^-DSA^-AAL^+$ fraction; *panel F*, the $ConA^-DSA^-AAL^-$ fraction. *Arrows a, b,* and *c* indicate the elution positions of authentic $Gal_4 \cdot GlcNAc_4 \cdot Man_3 \cdot GlcNAc \cdot Fuc \cdot GlcNAc_{OT}$, 2,4-branched $Gal_3 \cdot GlcNAc_3 \cdot Man_3 \cdot GlcNAc \cdot Fuc \cdot GlcNAc_{OT}$, and $Gal_3 \cdot GlcNAc_3 \cdot Man_3 \cdot GlcNAc \cdot Fuc \cdot GlcNAc_{OT}$, respectively. *Black triangles* indicate the elution positions of glucose oligomers (the *numbers* indicate the glucose units).

of the total asialo-oligosaccharides for rHuEPO and 87.4% for urinary HuEPO. The AAL-bound (AAL^+) fraction was also predominant in both samples: 85.6% for rHuEPO and 82.2% for urinary HuEPO, indicating that large parts of oligosaccharides are fucosylated at their core.

Each fraction was then analyzed by a column of Bio-Gel P-4. Since no qualitative difference in elution profile was detected between the two samples, only the results obtained from rHuEPO were shown in Fig. 2. Four fractions except for the DSA$^+$ fraction were eluted as single components (Fig. 2, *A–D*). The $ConA^-DSA^+AAL^+$ and AAL^- fractions were composed of four and two components, respectively (Fig. 2, *E* and *F*). The components thus separated were termed as AN1 to AN6 for the AAL^+ fractions and as AN1′ to AN4′ for the AAL^- fractions as shown in Fig. 2.

Structural Analysis of the Sugar Chains Obtained from rHuEPO and Urinary HuEPO—Structures of the oligosaccharides were studied by exoglycosidase digestions, Smith degradation, and methylation analysis as described in detail in the Miniprint Section of this paper and proposed as listed in Table II.

There was no qualitative difference in the structures of asialo-oligosaccharides between rHuEPO and urinary HuEPO. Both samples contain tetraantennary sugar chains (AN4 and AN4′) as major components: 46% for rHuEPO and 59.9% for urinary HuEPO. The tetraantennary sugar chains with *N*-acetyllactosamine repeating units (AN5 and AN6) were also detected in both samples, but their contents were much higher in rHuEPO (34.5%) than in urinary HuEPO (17.5%). The smaller portion of oligosaccharides (17.7% for rHuEPO and 32.6% of urinary HuEPO) had biantennary and 2,4-branched and 2,6-branched triantennary sugar chain structures.

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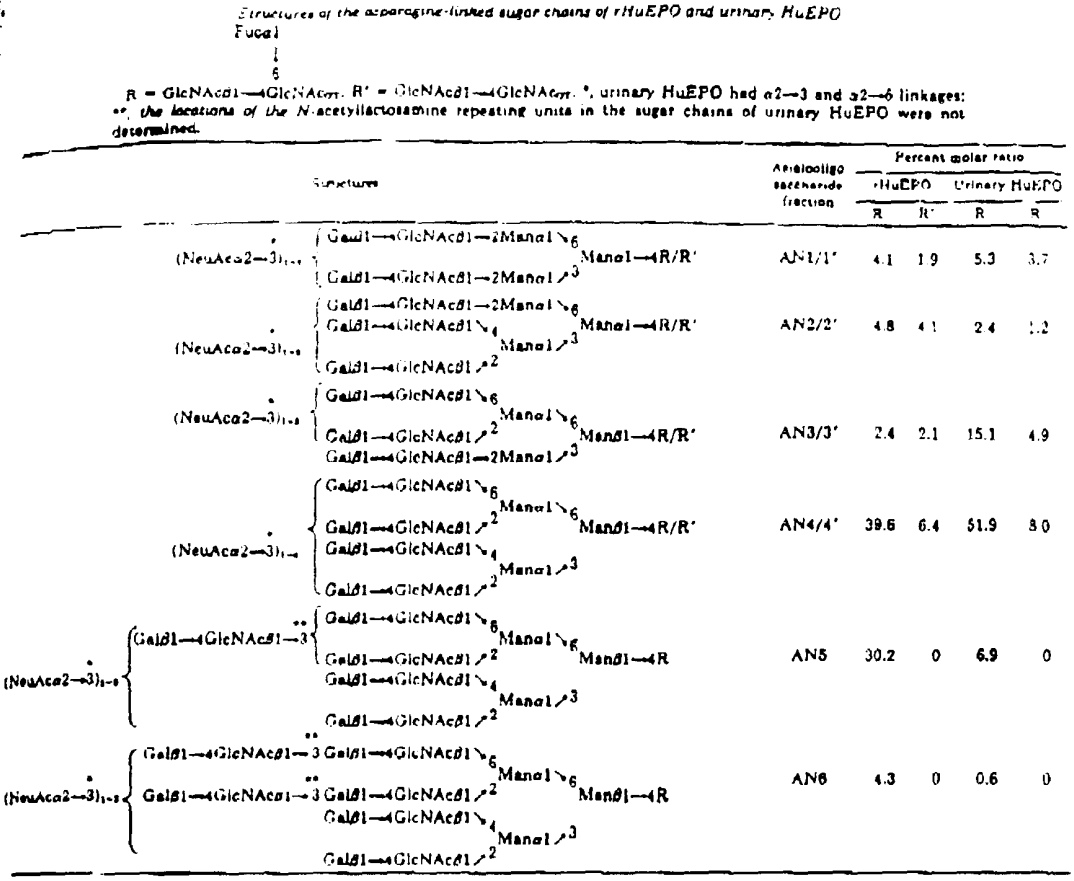
TABLE II

Structures of the asparagine-linked sugar chains of rHuEPO and urinary HuEPO



The number of sialic acid residues in each acidic component was analyzed as follows. When sialidase digests of the acidic fractions as shown in Fig. 1 were separately subjected to Bio-Gel P-4 column chromatography, it was shown that the tetraantennary oligosaccharide AN4 is included in the asialo-oligosaccharides of A-2, -3, -5, and -6, respectively (data not shown). Thus, the result and the mobility of each acidic fraction relative to authentic oligosaccharide standards indicated that one to four sites of AN4 are sialylated. Other oligosaccharides were also analyzed in the same way (data not shown). Methylation analysis of the oligosaccharide fraction of rHuEPO before (A) and after (AN) sialidase treatment indicated that almost all sialyl residues are linked at the C-3 position of galactosyl residues (Table III). This is in agreement with the fact that all the sialic acid residues of rHuEPO were susceptible to Newcastle disease virus sialidase (data not shown), which specifically cleaves the Siaα2—3Gal linkage. In contrast, approximately 40% of sialyl linkages were resistant to this viral sialidase in the case of urinary HuEPO (data not shown), indicating that other linkages are involved. Methylation analysis of oligosaccharide mixtures obtained from urinary HuEPO before and after sialidase treatment revealed that sialic acids in these oligosaccharides are linked at the C-3 and C-6 positions of galactose residues (data not shown). Therefore, the sialyl linkages resistant to the viral sialidase are considered to occur as the NeuAcα2—6Gal group.

DISCUSSION

Several glycoproteins have recently been produced by using recombinant techniques (15–17). To our knowledge, however, this is the first case to have analyzed comparatively the naturally occurring and the biotechnologically produced glycoproteins on the fine structural basis.

Both rHuEPO and urinary HuEPO contained tetraantennary oligosaccharides as major components. The residual oligosaccharides had biantennary and triantennary sugar chain structures. Both samples were also rich in fucosylated oligosaccharides. In addition to these similarities, some differences were also found in the sugar chains of the two erythropoietin samples. The total amount of complex-type oligosaccharides with *N*-acetyllactosamine repeating units in their outer chain moieties accounted for 34.5% in the case of rHuEPO, which was approximately five times higher than that of urinary HuEPO (7.5%). All sialic acid residues in the sugar chains of rHuEPO occur as the NeuAcα2—3Gal group, while about 60% of those of urinary HuEPO occur as the

TABLE III

Methylation analysis of acidic (A) and asialo-oligosaccharide (AN) fractions derived from rHuEPO

	Molar ratio*	
	A	AN
Fucitols		
2,3,4-Tri-*O*-methyl (1,5-di-*O*-acetyl)	0.9	0.9
Galactitols		
2,3,4,6-Tetra-*O*-methyl (1,5-di-*O*-acetyl)	0.8	3.3
2,4,6-Tri-*O*-methyl (1,3,5-tri-*O*-acetyl)	3.0	0.6
Mannitols		
2,4,6-Tri-*O*-methyl (1,2,5-tri-*O*-acetyl)	0.2	0.3
2,6-Di-*O*-methyl (1,2,4,5-tetra-*O*-acetyl)	0.9	0.9
3,4-Di-*O*-methyl (1,2,5,6-tetra-*O*-acetyl)	0.8	0.8
2,4-Di-*O*-methyl (1,3,5,6-tetra-*O*-acetyl)	1.0	1.0
2-*N*-Methylacetamido-2-deoxyglucitols		
1,3,5,6-Tetra-*O*-methyl (4-mono-*O*-acetyl)	0.2	0.2
3,6-Di-*O*-methyl (1,4,5-tri-*O*-acetyl)	5.0	5.0
1,3,5-Tri-*O*-methyl (4,6-di-*O*-acetyl)	1.0	1.0

*Numbers were calculated by taking the value for 2,4-di-*O*-methylmannitol as 1.00.

NeuAcα2—3Gal group and the others occur as the NeuAcα2—6Gal linkages. Despite these dissimilarities, the most important evidence is that all the oligosaccharides found in rHuEPO were included in urinary HuEPO. The absence of unusual sugar chains in rHuEPO is favorable for the clinical application of this hormone, since we do not need to take any account of antigenicity on its sugar moiety. It has been shown that sialidase digestion of urinary HuEPO results in the loss of its biological activity *in vivo* because of hepatic clearance (10, 11). Therefore, the fact that rHuEPO contained no neutral oligosaccharides might also be important for its clinical application.

The functional role of the sugar moiety of erythropoietin has not been resolved well, although its physiological significance was suggested by several studies. Sialic acid residues of erythropoietin are important not only for escaping from the hepatic clearance system of asialoglycoproteins but also may contribute to the conformational stabilization, since asialoerythropoietin becomes sensitive to heat denaturation and trypsin digestion (12). Desialylation does not decrease the *in vitro* activity of EPO but rather stimulates it when assayed at low concentration (12, 13). Thus, it is possible that the degree of sialylation affects the physical and biological properties of this glycoprotein. Recently, Dordal *et al.* (14) have shown that digestion of erythropoietin with endoglycosidase F or mixed glycosidases from *Diplococcus pneumoniae* results in the complete loss of its *in vivo* activity, but approximately 50% of its activity *in vitro* and immunoreactivity still remain. It has been suggested that the sugar chains are located at or near the binding domain of erythropoietin for its target cells (39). Therefore, it is likely that the sugar moiety of erythropoietin contributes to its biological function. Availability of the sufficient amount of rHuEPO and the structural information of its sugar moiety as obtained in this study will help us to resolve the functional roles of the sugar moiety of erythropoietin in the future.

Glycosylation of proteins primarily depends on the level of glycosyltransferases in the cells and is also affected by their primary amino acid sequences. The difference in sialyl linkages detected in the two erythropoietin samples may be the result of the former situation. In accordance with rHuEPO, G protein of vesicular stomatitis virus grown in CHO cells (44, 45) and recombinant γ-interferon produced in CHO cells (17) have been shown to contain only the NeuAcα2—3Gal linkage. Therefore, the exclusive expression of this sialyl linkage may indicate that CHO cells lack CMP-NeuAc:Galβ1—4GlcNAcα2—6sialyltransferase. All of the oligosaccharides detected in rHuEPO were also found in urinary HuEPO. This results also suggests the similarity of biosynthetic background between CHO cells and erythropoietin-producing cells in the human kidney. Alternatively, large parts of sugar chain structures may be regulated by the primary amino acid sequence of peptide. The major oligosaccharides of rHuEPO have tetraantennary structure, and considerable amounts of *N*-acetyllactosamine repeating structures were detected. However, such structures have been detected neither in γ-interferon produced in CHO cells (17) nor in vesicular stomatitis virus grown in CHO cells (40, 41). Instead, γ-interferon exclusively expressed biantennary structure, and vesicular stomatitis virus expressed biantennary and 2,6-branched triantennary structures. On the basis of these results, it seems likely that the primary amino acid sequence of polypeptide moiety is another important factor controlling the synthesis of sugar chains.

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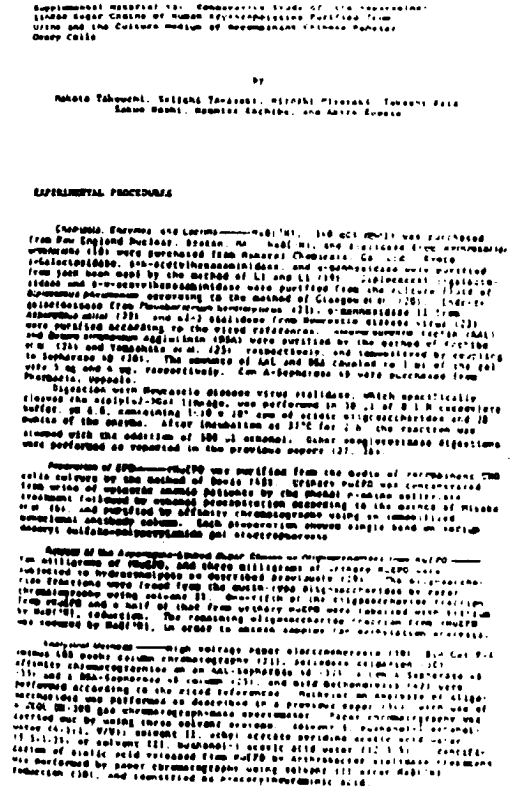

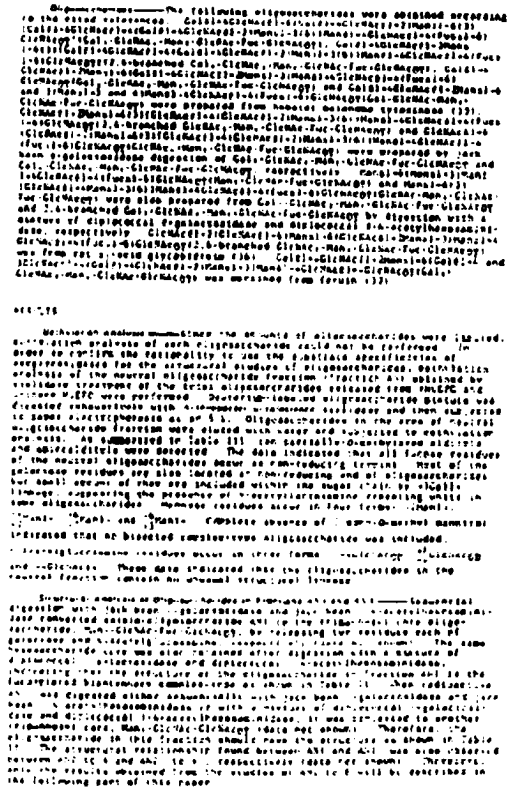

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