EXHIBIT J

Lodish Decl. in Support of Opposition to Roche's Motion for Summary Judgment of Invalidity for Double Patenting Over Claim 10 of the '016 Patent

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Biochemistry

Relationship between sugar chain structure and biological activity of recombinant human erythropoietin produced in Chinese hamster ovary cells

(N-linked oligosaccharide/role of carbohydrate moiety/mammalian cell/glycoprotein/hemopoietic hormone)

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ABSTRACT **Two forms of erythropoietin, EPO-bi and EPO-tetra, with different biological activities were isolated from the culture medium of a recombinant Chinese hamster ovary cell line, B8-300, into which the human erythropoietin gene had been introduced. EPO-bi, an unusual form, showed only one-seventh the *in vivo* activity and 3 times higher *in vitro* activity of the previously described recombinant human EPO (standard EPO). In contrast, EPO-tetra showed both *in vivo* and *in vitro* activities comparable to those of the standard EPO. EPO-bi, EPO-tetra, and the standard EPO had the same amino acid composition and immunoreactivity. However, structural analyses of their N-linked sugar chains revealed that EPO-bi contains the biantennary complex type as the major sugar chain, while EPO-tetra and the standard EPO contain the tetraantennary complex type as the major sugar chain. From examination of various preparations of recombinant human EPO, we found a positive correlation between the *in vivo* activity of EPO and the ratio of tetraantennary to biantennary oligosaccharides. These results suggest that higher branching of the N-linked sugar chains is essential for effective expression of *in vivo* biological activity of EPO.**

Human erythropoietin (EPO) is a glycoprotein hormone that plays a major role in regulating the level of circulating erythrocytes (1) by stimulating the maturation of late erythroid progenitor cells into proerythroblasts (2). In the normal human adult, EPO is produced in the kidney (3). EPO was first purified in a small amount from urine of aplastic anemia patients (4). Several recombinant human EPOs produced in mammalian (5–9) and nonmammalian cells (10) have recently become available, but their biological activities differ from cell to cell. Since the structures of their polypeptide moieties are the same, such variation in activity was suspected as being due to the differences in their carbohydrate moieties. The sugar chain structures of urinary human EPO and recombinant human EPO produced in Chinese hamster ovary (CHO) cells were determined independently by us (11) and by Sasaki *et al.* (12). Both urinary and recombinant human EPOs contain ≈40% carbohydrate in the form of three N-linked and one O-linked oligosaccharide chain. Important roles of the carbohydrate moiety in the solubility (13, 14), biosynthesis (14), and biological activity of EPO (9, 15) have been reported. Desialylation of EPO caused complete loss of its hormonal activity *in vivo* (15, 16) as the asialo-EPO was trapped in the liver (17) by the hepatic asialoglycoprotein binding protein (18) and was rapidly cleared from the circulation. Galactose oxidase treatment of asialo-EPO restored part of the biological activity (16). These results suggested that EPO possesses full biological activity only when it is sufficiently sialylated to avoid clearance by the hepatic asialoglycoprotein binding protein. During the course of study of the productivity of EPO in several recombinant CHO cell lines, we found a unique cell line, B8-300, which produced an unusual form of EPO (EPO-bi) in addition to the usual form (EPO-tetra). EPO-bi showed very little *in vivo* activity, even though it had a sufficient amount of sialic acid to cover most of its galactose residues. This finding suggested that sugar moieties other than sialic acid residues might be important for the expression of *in vivo* EPO activity. Therefore, we made a comparative analysis of the sugar chain structures and the hormonal activities of several preparations of recombinant human EPO in order to understand the relationship between oligosaccharide structure and biological activity of this hormone.

MATERIALS AND METHODS

Reagents and Samples. All of the EPOs used in this study were produced by recombinant CHO cells, which carried a gene encoding human EPO, as described (19) and were purified from the culture medium by a series of chromatographic procedures (20), including anion-exchange chromatography, reversed-phase chromatography, and gel filtration. In the case of EPO-bi, the reversed-phase chromatography step was not performed. The highly purified recombinant human EPO from a master cell line for which the N-linked sugar chains had been characterized (11) was used as a control and is called standard EPO in this paper. On an anion-exchange column, recombinant EPO produced by the B8-300 cell line was separated into two fractions: (*i*) EPO-tetra eluted at the same salt concentration and pH as the standard EPO, and (*ii*) EPO-bi eluted at a lower pH when a solution of 5 mM acetic acid and 6 M urea (pH 4.7) was used. Hydrazine was purchased from Aldrich and redistilled to remove water completely. NaB^3H_4 (319.0 mCi/mmol; 1 Ci = 37 GBq) was purchased from New England Nuclear. Other reagents were purchased from Nacalai Tesque, Kyoto.

Assay. The protein content of EPO was measured by radioimmunoassay (21) with a rabbit polyclonal antibody raised against standard EPO and EPO labeled with ^{125}I by the chloramine-T method (22). The *in vitro* and *in vivo* biological activities of the various recombinant human EPO preparations were assayed by incorporating ^{59}Fe into rat bone marrow cells in culture (23) and into erythroblast cells of exhypoxic polycythemic mice (24), respectively. The highly purified recombinant human EPO calibrated by the Second

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Abbreviations: EPO, erythropoietin; TN, total neutral oligosaccharides.

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Table 1. Specific activities of EPO-bi, EPO-tetra, and standard EPO

Assay method	Specific activity, IU per mg of EPO		
	EPO-bi	EPO-tetra	Standard EPO
Polycythemic mouse (*in vivo*)	19,500	111,000	134,000
Bone marrow culture (*in vitro*)	397,000 ± 12,900	138,000 ± 39,500	134,000 ± 7600

Each *in vivo* value was determined by a parallel line assay method (27) using three doses per sample and three mice per dose. The interassay coefficient of variation was 15%. Each *in vitro* value is the mean ± SEM of six experiments. IU, international unit for EPO.

International Reference Preparation was used as standard in all assays.

Sugar Chain Analysis. The structural analysis of sugar chains was carried out as described in our previous report (11). Briefly, N-linked oligosaccharide chains of EPO were released from protein by hydrazinolysis and radiolabeled by NaB^3H_4 reduction (25). The number of charges of the oligosaccharides was analyzed by FPLC using a Mono Q HR5/5 column (Pharmacia, Uppsala). The branching structures of the oligosaccharides were analyzed after desialylation with sialidase from *Arthrobacter ureafaciens* (Nacalai Tesque) by gel-permeation chromatography with Bio-Gel P-4 (−400 mesh; Bio-Rad) (26).

RESULTS AND DISCUSSION

Structural Analysis of Sugar chains of EPO-bi and EPO-tetra. The EPO-tetra showed *in vivo* specific activity comparable to that of the standard EPO, while the EPO-bi showed only one-seventh the *in vivo* specific activity of standard EPO (Table 1). In contrast, the specific activity obtained by an *in vitro* assay of EPO-bi was 3 times higher than that of the standard EPO. Both EPO-bi and EPO-tetra showed exactly the same amino acid compositions, while isoelectric focusing analysis indicated that EPO-bi contained a series of isoforms less negative than those found in EPO-tetra and the standard EPO (data not shown). Then, we analyzed their N-linked sugar chains liberated by hydrazinolysis. When the released oligosaccharide mixtures were subjected to FPLC with a Mono Q column, they were separated into N, A1, A2, A3, and A4 with 0, 1, 2, 3, and 4 negative charges, respectively (Fig. 1). The percent molar ratios of these fractions were as follows: 12, 22, 35, 22, and 9 in EPO-bi; 3, 10, 25, 32, and 30 in EPO-tetra. The values for standard EPO were 1, 14, 29, 33, and 23. As described in a previous report (11), the acidic oligosaccharide fractions were almost completely converted by sialidase treatment to neutral ones (data not shown). Therefore, the difference in elution profiles of FPLC indicated that EPO-bi contains relatively less sialylated oligosaccharides than EPO-tetra or the standard EPO.

To analyze the neutral portions of the oligosaccharides, the asialo-oligosaccharides obtained by sialidase digestion of A1, A2, A3, and A4 were combined with neutral oligosaccharides, and the total neutral oligosaccharides (TN) thus obtained were subjected to Bio-Gel P-4 column chromatography (Fig. 2). The oligosaccharides from both EPO-bi (Fig. 2*A*) and EPO-tetra (Fig. 2*B*) were fractionated into five components, but their ratios were quite different. The elution profile obtained from the standard EPO was similar to that of EPO-tetra (data not shown). The percent molar ratio of TN1, TN2, TN3, TN4, and TN5 obtained from each sample was as follows: 36/30/23/8/3 for EPO-bi, 14/14/58/10/4 for EPO-tetra, and 6/14/46/30/4 for the standard EPO. As docu-

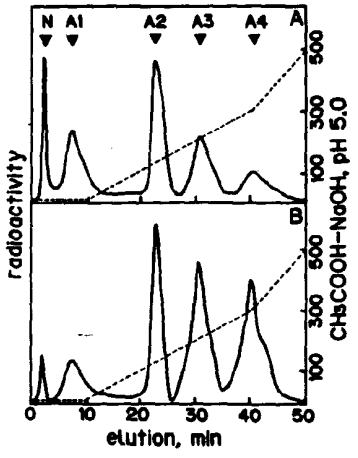

FIG. 1. Charge analysis of N-linked oligosaccharides obtained from EPO-bi and EPO-tetra. ^3H-labeled oligosaccharides were subjected to FPLC using a Mono Q HR 5/5 column equilibrated with 5 mM sodium acetate buffer (pH 5.0) and eluted with a linear gradient of the same buffer (5–500 mM) at 1 ml/min. (*A* and *B*) The N-linked oligosaccharides of EPO-bi and EPO-tetra, respectively. Arrowheads N, A1, A2, A3, and A4 indicate the elution positions of oligosaccharides containing 0, 1, 2, 3, and 4 negative charges (sialic acids), which were obtained from the standard EPO.

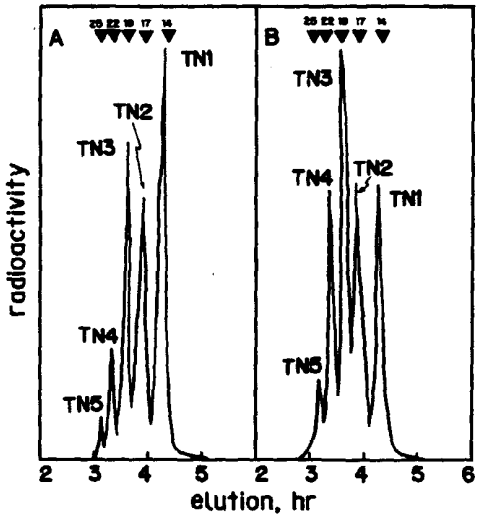

FIG. 2. Analysis of fraction TN on a Bio-Gel P-4 column. The mixture of ^3H-labeled neutral and asialo-oligosaccharides was fractionated on a column containing Bio-Gel P-4 (−400 mesh; 2 × 100 cm) equilibrated with deionized distilled water at 0.5 ml/min. (*A* and *B*) The elution patterns of oligosaccharide fractions obtained from EPO-bi and EPO-tetra, respectively. Arrowheads indicate the elution positions of the standard glucose oligomers and numbers indicate glucose units.

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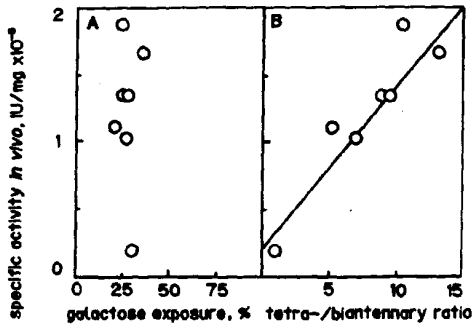

FIG. 4. Relationship between oligosaccharide structure and *in vivo* specific activity of recombinant human EPO. The specific activity was expressed as *in vivo* activity per mg of EPO. The equations to calculate the extent of galactose exposure and the tetraantennary/biantennary ratio are described in the text. (*A*) Relationship between galactose exposure and the *in vivo* specific activity of EPO. (*B*) Relationship between the ratio of tetraantennary to biantennary oligosaccharides and the *in vivo* specific activity of EPO.

branching structure of the sugar chains influences the biological activity of this glycoprotein hormone.

Sugar chains of glycoprotein hormones may play important roles in (*i*) allowing the proteins to achieve the correct conformation during biosynthesis (28), (*ii*) allowing secretion from cells without being trapped or degraded (14), (*iii*) conferring an adequate half-life in serum by affording protection from trapping by hepatic binding protein or degradation by proteases (14), and (*iv*) activation of a biological response once bound to the receptor of target cells (29). Removal of a glycosylation site by site-directed mutagenesis has been observed to impair the secretion of several glycoproteins (30, 31), including EPO (14).

The low *in vivo* activity of EPO-bi is not due to a defect in the activation of the biological response at the receptor level, since the EPO-bi demonstrated high activity in the *in vitro* assay. This is in contrast to the fact that deglycosylated forms of the pituitary and placental glycoprotein hormones cannot activate their target cells *in vitro* (29, 32, 33). There may be a barrier against EPO-bearing small sugar chains on the way to the target tissue. The observation that the *in vitro* activity of EPO-bi is higher than that of the standard EPO is probably due to its lower overall charge (due to lower total sialic acid content), which might facilitate the approach to a receptor. This has been postulated for human lutropin and human chorionic gonadotropin (34) from the observations that their desialylated forms show increased binding to their target cells.

Several explanations can be made for the higher *in vivo* activity of human EPO-bearing tetraantennary oligosaccharide chains. The greater activity could be due to the larger size and/or greater total negative charge of the EPO-tetra lessening some nonspecific clearance mechanism such as filtration by the kidney or interaction with serum components such as proteases or binding proteins. Alternatively, the tetraantennary sugar chains could aid in the sequestration or retention of EPO in the target tissue through their structure, size, charge, or some specific interaction.

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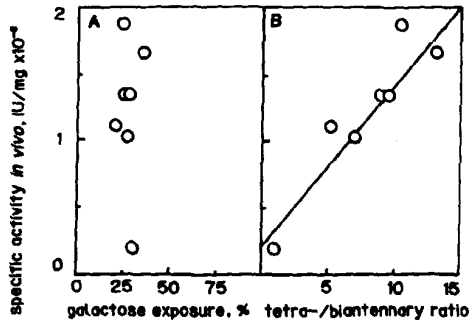

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