Role of Sugar Chains in the Expression of the Biological Activity of Human Erythropoietin*

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Various deglycosylated derivatives of recombinant human erythropoietin (hEPO) were prepared and used to determine the role of the sugar chains in the expression of its biological activity in vivo and in vitro. Three N-linked oligosaccharides of hEPO have been partially or fully removed to obtain N-glycan (NG) (2)-, NG(1)-, and NG(0)-hEPO carrying two, one, and no N-linked sugar chains, respectively. The preparation lacking only O-linked sugar chain O O-glycan (OG) (0)-hEPO was also used. As de-N-glycosylation proceeded, the in vivo activity of the hormone decreased drastically, and the activity of these derivatives was correlated with the number of sialic acids bound to them. On the contrary, the in vitro activity was increased by the de-N-glycosylation; NG(0)-hEPO showed a 3-fold higher specific activity than the intact hormone. This was confirmed by binding experiments of the derivatives to target cells. The in vitro activity and the affinity also correlated with the number of sialic acids bound to the deglycosylated hEPO preparations. On the other hand, OG(0)-hEPO was as active as the intact hormone in vivo and in vitro. In conclusion, the N-linked sugar chains are not required for in vitro activity but required for in vivo activity, acting as anchors for the essential terminal sialic acids. The O-linked sugar chain has no essential role in the biological activity of the hormone in vivo or in vitro.

Erythropoietin (EPO), a sialoglycoprotein primarily produced in the kidney of adults and in the liver of the fetus (1-3), plays a major role in the proliferation of and differentiation of erythroid progenitor cells to erythrocytes (4). Human EPO (hEPO) carries three N-linked and one O-linked oligosaccharide chains attached at Asn-24, -38, and -83, and at Ser-126, respectively (5). The structure of N- and O-linked carbohydrate of hEPO produced in Chinese hamster ovary cells or baby hamster kidney cells, to which a cDNA clone for hEPO was transfected, has been reported (6-8). These studies show that the major carbohydrate chain of the hormone is sialylated tetraantennary oligosaccharides with or without N-acetyllactosaminyl repeats. The importance of the terminal sialic acid residues for the expression of the biological activity in vivo and in vitro has been reported using crude EPO preparations (9-12); the sialic acids are essential in vivo but not required in vitro. The complete loss of hEPO activity by desialylation in vivo has been confirmed if it is a result of hepatic removal of the asialo-hEPO from circulation (13, 14).

We have demonstrated in a previous paper (15), using highly purified recombinant hEPO, that the asialo-hEPO shows a 3-6-fold higher specific activity than the intact hormone in vitro and that the increased activity is caused by the increase of affinity between asialo-EPO and EPO receptors (15). We have also shown, by using various partially or fully desialylated hEPO, that the in vivo activity of the hormone fully depends on the number of sialic acid residues.

Recently, Tsuda et al. (16) and Takeuchi et al. (17) have reported the biological activity of hEPO in which three N-linked sugar chains had been fully removed. They have shown that the in vivo activity of fully de-N-glycosylated hEPO is completely lost. However, the significance of the sugar chains in the expression of in vitro activity remains confused. Tsuda et al. showed that the in vitro activity remained after removal of N-linked or total sugar chains, but Takeuchi et al. showed that the activity in vitro is completely abolished. This contradiction may be derived from not having isolated the deglycosylated derivatives and not fully characterizing them physicochemically. Before evaluation of the biological activity, it is essential to confirm that the deglycosylated derivatives have not been damaged in the polypeptide moiety, as determined by physicochemical characterization. In this paper, we describe the contribution of N-linked and O-linked oligosaccharide chains to the biological activity of hEPO in vivo and in vitro using the deglycosylated preparations fully characterized physicochemically. We also discuss the site-specific N-glycosylation of hEPO analyzed by sugar mapping.

EXPERIMENTAL PROCEDURES AND RESULTS

We have successfully clarified the role of the N-linked and O-linked sugar chains in the biological activity of hEPO using various deglycosylated forms of hEPO. The preparations used were NG(2)-, NG(1)-, and NG(0)-hEPO carrying two, one, and no N-linked sugar chains, respectively (Fig. 1). We also used the intact hEPO as a control, OG(0)-hEPO (lacking only

* Portions of this paper (including "Experimental Procedures," Figs. 1-8, and Tables I-III) 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.
O-linked sugar chain but containing three N-linked sugar chains), and the fully deglycosylated hEPO. Physicochemical analyses demonstrate that undesirable degradation did not occur during preparation of the sample (Figs. 2-4, and Tables I and II). In conclusion, N-linked sugar chains are essential for the expression of the biological activity of hEPO in vivo, but their role is restricted to a role as anchors for the terminal sialic acids. On the other hand, the N-linked sugar chains are not required for the in vitro activity, and the O-linked sugar chain does not have affect any on either activity. The significance of sialic acids bound to the O-linked sugar chain is thought to be different from that bound to the N-linked sugar chains.

In Vivo Activity—The importance of the N-linked sugar chains in the in vivo activity of hEPO has been confirmed; as de-N-glycosylation proceeded, the in vivo activity of the hormone decreased drastically (Table IV). Results obtained here indicate that all the N-linked sugar chains contribute almost equally to the in vivo activity, and that there are no specifically important N-linked sugar chain. A linear relationship between the logarithm of in vivo activities of partially and fully de-N-glycosylated hEPO and the number of sialic acids bound to them was obtained. If a specific N-linked sugar chain contributed to the activity, either NG(2)- or NG(1)-hEPO would not have been shown an in vivo activity correlated with the number of sialic acids.

We have also clarified the role of the O-linked sugar chain of the hormone in vivo using OG(0)-hEPO. This showed almost the same specific activity as the intact hEPO, indicating that the O-linked sugar chain has no effect in the expression of the in vivo activity. However, this lack of contribution of the O-linked sugar chain was assessed in the presence of the N-linked sugar chains. The presence of the N-linked sugar chains may mask the function of the O-linked sugar chain. Then, we compared the activities of NG(0)-hEPO, which carries one O-linked sugar chain but no N-linked sugar chains, asialo-NG(0)-, and fully deglycosylated hEPO, NG(0)-, asialo-NG(0)-, and fully deglycosylated hEPO showed in vivo activities of 800, 600, and 300 units/mg, respectively, although these values were quite low (0.14–0.38%) when compared with that of the intact hEPO (2.13 × 10^7 units/mg). From these results, in terms of the role of sugar chains in the in vivo activity, it is concluded that the three N-linked sugar chains of hEPO are important, being required as anchors for the essential terminal sialic acids, but the O-linked sugar chain does not contribute. The O-linked sugar chain carries about 1.5 mol of sialic acid. However, the biological significance of sialic acids bound to the O-linked sugar chain is thought to be different from those bound to the N-linked sugar chains because NG(0)- and asialo-NG(0)-hEPO showed almost the same specific activity.

The loss of the in vivo activity of asialo-hEPO is explained by the hepatic removal from circulation; however, a portion of the derivative may escape from capture via the lectin in the liver to still reach bone marrow. It is widely believed that asialo-hEPO and de-N-glycosylated hEPO completely lose their in vivo activity. Even fully desialylated hEPO showed an in vivo activity, which, however, is only 0.1–0.2% that of the intact hEPO. The question is how partially or fully de-N-glycosylated hEPO are removed from circulation. We can not explain this phenomena satisfactorily at present, but we have some data that the disposition of de-N-glycosylated hEPO is different from asialo-hEPO. It has been found that the de-N-glycosylated hEPO are uptaken and degradable mainly in the kidney but not in the liver. The half-life of NG(2)-hEPO is almost the same as the intact hEPO, but further de-N-glycosylation caused shorter half-lives.

In Vitro Activity—The role of the N-linked sugar chains of hEPO in vitro is quite different from that of in vivo. Results shown in Figs. 6 and 7 and Table IV demonstrate that the derivatives have a higher specific in vitro activity than the intact hormone. In disagreement with these results, however, Takeuchi et al. (17) have reported that N-glycanase digestion of the intact hEPO resulted in almost a complete loss of the activity in vitro. They have concluded that the core portion of the Asn-type sugar chain is necessary for erythropoietin to have its full biological activity in vitro and suggest that removal of the core portion of the sugar chains destroys the active conformation of erythropoietin. Their results are quite opposite to those that we have obtained. We used the hEPO derivatives after they had been fully characterized. Furthermore, de-N-glycosylated hEPO showed almost the same CD spectra, indicating that full de-N-glycosylation does not affect to the structure of hEPO even though up to about 40% total mass of the hormone had been removed (Fig. 4). The binding ability of the fully deglycosylated hEPO to human erythroblastic cells increased. Therefore, it is reasonable to think that the N-linked sugar chains of hEPO are not required for the in vitro activity.

Binding activity of the deglycosylated hEPO to FMLC was evaluated by competition for the iodolabeled intact hEPO using the AccuFit Competition program. Affinity between the deglycosylated hEPO and the specific receptors for hEPO increased by de-N-glycosylation, but the number of binding sites was unchanged. The ratio of the Kd values and the half-maximal doses for CFU-E colony-formation of de-N-glycosylated hEPO to the intact hEPO showed a good accordance (Table IV and Fig. 8). OG(0)-hEPO showed almost the same Kd value as the intact hormone. These results demonstrated that the higher specific activities of the de-N-glycosylated hEPO depend on the affinity between it and the receptors. Why then is the affinity between hEPO and its receptors increased by deglycosylation? One possible explanation is that the negative charges of the intact hormone carried by the

TABLE IV
Summary of the biological activity of the deglycosylated forms of hEPO

| hEPO sample | No. of sugar chain | In vivo activity | In vitro activity | Binding to FMLC |
|-------------|--------------------|-----------------|------------------|----------------|
|             | N-linked | O-linked | units/mg × 10^-4 | units/mg × 10^-4 | EDso (pg/ml) | Kd | Binding site |
| Intact      | 3        | 1        | 21.3             | 28.5             | 22.2         | 320 | 470 |
| NG(2)      | 2        | 1        | 4.1              | 33.8             | 17.2         | 250 | 500 |
| NG(1)      | 1        | 1        | 1.4              | 40.5             | 12.3         | 150 | 520 |
| NG(0)      | 0        | 1        | 0.08             | 50.9             | 9.0          | 120 | 530 |
| OG(0)      | 3        | 0        | 22.0             | 27.7             | 22.6         | 320 | 490 |

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sialic acids inhibit the interaction between the hormone and the receptors. When the affinity between the FMLC and 125I-hEPO or 125I-asialo-hEPO was determined by Scatchard analysis, the affinity was increased but the binding sites per cell were not changed by desialylation. Furthermore, the intact hEPO showed almost the same affinity to the neuraminidase-treated FMLC as asialo-hEPO. The intact hEPO has a high negative charge (pI = 3.0–4.2) but the deglycosylated hEPO does not. Therefore, the explanation that the negative charges of the sialic acids inhibit the interaction between the hormone and its receptors can be applicable to the deglycosylated hEPO.

Site-specific N-Glycosylation—hEPO carries three N-linked sugar chains (N24, N38, and N83) attached at Asn-24, -38, and -83. The Asn-83 sugar chain had the highest susceptibility to glycopeptidase F out of the three sugar chains because NG(2)-hEPO lacks only the Asn-83 sugar chain but has the Asn-24 and -38 sugar chains intact. The Asn-24 and -38 sugar chains have a similar susceptibility to the enzyme because NG(1)-hEPO has been shown to consist of an equal amount of the derivatives that carry the Asn-24 or -38 sugar chains. This may be explained by the two glycosylation sites being closely located to one another.

Recombinant hEPO has been reported to carry over 10 different kinds of N-linked sugar chains as does human urinary EPO (6–8). We have analyzed site-specific N-glycosylation to determine the structure of certain sugar chains to see if these certain sugar chains are essential for the expression of the biological activity of hEPO in vivo. We have previously reported site-specific glycosylation of the hormone using fast atom bombardment mass spectrometry and reported that the sugar chains attached to Asn-83 are composed mainly of tetraantennary without N-acetyllactosaminyl repeats (32). However, now we demonstrate that the Asn-83 sugar chain is as heterogeneous as the Asn-38 sugar chain by the sugar mapping employed here (See Fig. 5 and Table III). The difference is derived from the difference of the peptide samples analyzed. The glycopeptide K4, containing the Asn-83 sugar chain, shows multiple peaks close together on the peptide map (see Fig. 3a). This is caused by the heterogeneity of the sugar chains. In our previous study, we used the major peak of K4, but have used all peaks in the present study. The N-linked sugar chains were obtained by enzymatic digestion of the hormone using glycopeptidase F, not by chemical cleavage of hydrazinolysis. An advantage of the enzymatic method is that the degree of release of the sugar chain can be easily monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis since the protein moiety remains intact. The other advantage of sugar mapping using reverse-phase HPLC and ion suppression amine adsorption-HPLC is that once the structure of each peak has been identified, we can estimate the structure of the sugar chains with a high probability by simple HPLC.

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Deglycosylation of hEPO— Recombinant hEPO (50 mg) was partially digested with 20 units of Glycopeptidase F from Flavobacterium meneghinianum (Boehringer Mannheim) in 3.5 ml of 0.5 M sodium phosphate buffer, pH 7.4, containing 5 mM NaCl and 0.2% Tween 20 at 24°C for 3 h. The resulting digest was a mixture of hEPO, in which three N-linked sugar chains were partially removed, and PA oligosaccharides carrying two, or three N-linked sugar chains are termed NG2, NG3, and NG4-hEPO, respectively. The digest was applied to a 4.6 × 250 mm Vydac protein C4 reverse-phase HPLC column equilibrated with 50 mM sodium phosphate buffer, pH 7.4 containing 0.1 M NaCl and 0.02% Tween 20. The column was developed with the same buffer at a flow rate of 1.0 ml/min, and the N-linked sugar chains were eluted with a decreasing linear gradient of 0.01% trifluoroacetic acid (2% acetonitrile for 15 min) to 50% acetonitrile (80% acetonitrile in 3% trifluoroacetic acid for 15 min). The eluate was monitored at 205 nm. The PA-oligosaccharides were digested with 2.5 units of Endoproteinase Glu-C (Vydac Peptide C18) dissolved in 10 ml of 0.25 M ammonium bicarbonate, pH 8.3, and the modified hEPO was subjected to size fractionation HPLC, using a Vydac protein C4 reverse-phase HPLC column (4.6 × 250 mm) equilibrated with 80% acetonitrile in 3% trifluoroacetic acid (25 mM ammonium acetate buffer, pH 6.0) containing 0.1% trifluoroacetic acid. The PA-oligosaccharides were subjected to tryptic digestion in the glycopeptide moiety with 5 units of TPCK-trypsin (250 mM ammonium bicarbonate, pH 8.3, and 50 mM acetic acid) for 2 h at 37°C. The diglycosylated derivatives were eluted with a linear gradient of acetonitrile from 0% to 50% for 10 min, in 0.1% trifluoroacetic acid, and the modified hEPO was purified by reverse-phase HPLC as described above. hEPO lacking the O-linked sugar chains was purified according to the method described previously except that the 100-kDa fraction was used for MALDI-TOF mass spectrometric analysis for amino acid composition analysis.

I-isolation of N-linked oligosaccharides— Azoal hEPO (500 mg) prepared by neuraminidase digestion of the hormone and purified by reverse-phase HPLC as described above, was applied to a 4.6 × 250 mm Vydac protein C4 reverse-phase HPLC column (4.6 × 250 mm) equilibrated with 0.01% trifluoroacetic acid and 0.1% acetic acid in water to elute the N-linked sugar chains, and the HPLC fractions were collected every 15 s.

Isolation of N-linked oligosaccharides from each glycosylation site— Recombinant hEPO (3.0 mg) was treated with PNGase F and PA-oligosaccharides were isolated by reverse-phase HPLC as described above. The N-linked sugar chains were purified by neuraminidase digestion and Endoproteinase Lys-C (Boehringer Mannheim) as described previously. Glycosylation, K2 and K4 carrying N2 and N4, and NG4 sugar chains, respectively, were eluted by reverse-phase HPLC using a Vydac C18 column (4.6 × 250 mm) as described above. K1 and K2V1, KZV2 and K4V3 were digested with additional 5 units of PNGase F for 2 h at 37°C. The diglycosylated derivatives were digested with 2.5 units of Endoproteinase Glu-C (Vydac Peptide C18) dissolved in 10 ml of 0.25 M ammonium bicarbonate, pH 8.3, and the modified hEPO was subjected to size fractionation HPLC, using a Vydac protein C4 reverse-phase HPLC column (4.6 × 250 mm) equilibrated with 80% acetonitrile in 3% trifluoroacetic acid (25 mM ammonium acetate buffer, pH 6.0) containing 0.1% trifluoroacetic acid. The PA-oligosaccharides were subjected to tryptic digestion in the glycopeptide moiety with 5 units of TPCK-trypsin (250 mM ammonium bicarbonate, pH 8.3, and 50 mM acetic acid) for 2 h at 37°C. The diglycosylated derivatives were eluted with a linear gradient of acetonitrile from 0% to 50% for 10 min, in 0.1% trifluoroacetic acid, and the modified hEPO was purified by reverse-phase HPLC as described above.
contribution of the 0-linked sugar chain

deglycosylated hEPO

dominant

9.

intact hEPO used hEPO. On the other hand, OG(0)-hEPO was as active that the 0-linked hEPO

activity.

and fully deglycosylated hEPO showed specific activities deglycosylated hEPO than NG(0)-hEPO.

Binding

Tnbc IV

shown in Fig. 6. Partially number of hEPO preparations comparing with the intact hormone are summarized number when desialylated hEPO.

and 3.84 I@ units/mg. respectively. The biological activity of various the intact hEPO. The ratio of the kd values and the de-N-glycosylated hEPO also showed a three-times-higher specific activity than the intact form. Fully deglycosylated hEPO showed the same in both forms of hEPO; 110 and 130 sites/cell for the intact and for the fully deglycosylated hEPO, respectively.

Binding of deglycosylated hEPO to cells——The increase of the activity in vivo with de-N-glycosylation is thought to be caused by the increase in affinity between the derivatives and the specific receptors for hEPO expressed on the surface of the target cells. Therefore, various deglycosylated preparations were examined in the binding assay using hEPO. Competition analyses performed in the presence of 1hEPO demonstrated that the kd values lowered as de-N-glycosylation proceeds, but the number of sites per cell was unchanged. Biological activities of the de-N-glycosylated hEPO preparations comparing with the intact hormone are summarized in Table IV. Table IV also shows that the OG(0)-hEPO showed almost the same kd and the same number of sites as the intact hEPO. This was also good as a correlation between the kd value and the activity in the deglycosylation formation as did NG(0)-hEPO. These results demonstrate that the increase of the biological activity in vivo with de-N-glycosylation depends on the higher affinity between the derivatives and the EPO receptors. Similar results had been obtained with fully deglycosylated hEPO was examined as reported previously(15). Fig. 8 shows a correlation between the half-maximal doses in the EPO-formation column and the number of sialic acids bound to the intact and the de-N-glycosylated forms of hEPO. A correlation between the kd values and the number of sialic acids is also shown. A good linear correlation was obtained in both forms. Fully deglycosylated hEPO also showed a higher affinity than the intact hEPO when they were binding assay using human erythroleukemia cells. Kd values were 250 and 60 pM for the intact and the fully deglycosylated hEPO, respectively. The number of sites per cell was almost the same in both forms of hEPO; 110 and 130 sites/cell for the intact and for the fully deglycosylated hEPO, respectively.

Role of Sugar Chains in Human Erythropoietin

In vivo activity of deglycosylated hEPO——The biological activity of various deglycosylated hEPO derivatives was determined in vivo by the 1hEPO-incorporation assay. OG(0)-hEPO showed almost the same specific activity as the intact hEPO, demonstrating that the 0-linked sugar chain of the hormone does not contribute to the in vivo activity of the hormone, as in the case with in vivo activity. The significance of the 0-linked sugar chain can be evaluated by comparing the activities of NG(0)- and fully deglycosylated hEPO. The two forms of hEPO showed the specific activities of 5.09 x 105 and 3.84 x 105 units/mg, respectively. This lower specific activity of the fully deglycosylated hEPO than NG(0)-hEPO, but still higher than that of the intact hEPO, may be derived from its instability.

In the other hand, as de-N-glycosylation proceeds, the activity increases; the specific activities of the intact, NG(0)-, and NG(0)-hEPO were 2.85, 3.38, 4.05 and 5.15 x 105 units/mg, respectively. These results, i.e., that de-N-glycosylation causes an increase of the in vivo activity, were confirmed by CFE-colony-formation assay as shown in Fig. 6. Partially or fully de-N-glycosylated hEPO showed an enhanced activity. In particular, NG(0)-hEPO showed a three-times-higher specific activity than the intact form. Half-maximal doses for the CFE-colony-formation were 22.2, 17.2, 12.3 and 9.0 pg/mg for the intact, NG(0)-, NG(0)- and fully deglycosylated hEPO, respectively.

Binding of deglycosylated hEPO to cells——The increase of the activity in vivo with de-N-glycosylation is thought to be caused by the increase in affinity between the derivatives and the specific receptors for hEPO expressed on the surface of the target cells. Therefore, various deglycosylated hEPO preparations were examined in the binding assay using hEPO. Competition analyses performed in the presence of 1hEPO demonstrated that the kd values lowered as de-N-glycosylation proceeds, but the number of sites per cell was unchanged. Biological activities of the de-N-glycosylated hEPO preparations comparing with the intact hormone are summarized in Table IV. Table IV also shows that the OG(0)-hEPO showed almost the same kd and the same number of sites as the intact hEPO. The ratio of the kd values and the number of sites as the intact hEPO. The ratio of the kd values and the half-maximal doses for the CFE-colony-formation of NG(0)-hEPO to that of the intact form were in good accordance. 2.8 for the kd and 2.5 for the CFE-colony-formation. NG(0)- and NG(0)-hEPO also showed as good a correlation between the kd value and the activity in the EPO-formation column as did NG(0)-hEPO. These results demonstrate that the increase of the biological activity in vivo with de-N-glycosylation depends on the higher affinity between the derivatives and the EPO receptors. Similar results had been obtained with fully deglycosylated hEPO was examined as reported previously(15). Fig. 8 shows a correlation between the half-maximal doses in the CFE-colony-formation and the number of sialic acids bound to the intact and the de-N-glycosylated forms of hEPO. A correlation between the kd values and the number of sialic acids is also shown. A good linear correlation was obtained in both forms. Fully deglycosylated hEPO also showed a higher affinity than the intact hEPO when they were binding assay using human erythroleukemia cells. Kd values were 250 and 60 pM for the intact and the fully deglycosylated hEPO, respectively. The number of sites per cell was almost the same in both forms of hEPO; 110 and 130 sites/cell for the intact and for the fully deglycosylated hEPO, respectively.

Fig. 1. Separation of de-N-glycosylated forms of hEPO by gel-permeation chromatography. Highly purified recombinant hEPO (0.5 mg) was partially digested with 20 units of Glycosidase P in 3.5 ml of 0.2 M sodium phosphate buffer, pH 8.4, containing 5 mM EDTA, for 24 h at 37°C. The resulting digest was applied to a TSK G3000SW column (25 x 600 mm) equilibrated with 50 mM sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl and 0.05% Tween 20. The column was developed with the same buffer at a flow rate of 0.75 ml/min, and proteins were monitored at 280 nm. The resulting three fractions a, b, and c, corresponding to NG(2), NG(1), and NG(0)-hEPO, respectively, were pooled.

Fig. 2. SDS-PAGE of the deglycosylated forms of hEPO. Samples (about 2 ,ug) were electrophoresed on 13.5% gel and stained with Coomassie brilliant blue R-250. Molecular weight markers used were phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and lysozyme (Bio-Rad). Lanes 1 and 8, molecular weight markers; lane 2, intact hEPO; lane 3, OG(0)-hEPO; lane 4, NG(0)-hEPO; lane 5, NG(0)-hEPO; lane 6, NG(0)-hEPO; lane 7, fully deglycosylated hEPO.

Fig. 3. Peptide maps of the deglycosylated forms of hEPO. Each sample (50 ,ug) in 200 ml of 0.1M NaCl/EDTA, pH 8.4, was digested with Endoproteinase Lys-C for 24 h at 37°C and at a substrate/enzyme ratio of 20. After addition of 20 ml of 10% trifleuoroacetic acid, the digest was applied to a Vydac Peptide C18 reverse-phase column (4.6 x 250 mm) equilibrated with 10% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 1.0 ml/min. Peptides were eluted with a series of the linear gradient of acetonitrile from 10 to 80% over 140 min and detected by their absorbance at 220 nm. The glycosylated peptides K2, K4, and K6 were subjected to amino acid sequence analysis. The partially deglycosylated K2 shows as K2, and the deglycosylated K2, K4, and K6 shows as K2', K4' and K6', respectively, were also analyzed. a, intact hEPO; b, NG(2)-hEPO; c, NG(1)-hEPO; d, NG(0)-hEPO; e, OG(0)-hEPO.
Fig. 4. CD spectra of the deglycosylated forms of hEPO. CD spectra were taken in a Jasco J-715A recording spectropolarimeter equipped with a DP-500N data processor at a protein concentration of 50 μg/ml in 50 mM sodium phosphate buffer, pH 7.4, containing 0.15M NaCl and 0.02% Tween 20. The conditions employed were: scan speed, 20 nm/min; sensitivity, 2 μV/cm; time constant, 1 sec; accumulation time, 8; light path length, 2 mm; temperature, 21°C. The CD spectrum of OG(0)-hEPO was identical to that of the intact hEPO. ——, intact hEPO; ——, NG(2)-hEPO; ———, NG(1)-hEPO; ——, NG(0)-hEPO.

Fig. 5. Analysis of site-specific N-glycosylation. PA-antialloosaccharides were subjected to a Shimpack CLC-Dex column (6 × 250 mm) equilibrated with 0.01% trifluoroacetic acid in 10 mM sodium phosphate buffer, pH 3.8, at a flow rate of 1.4 ml/min and at 4°C. The saccharides were eluted with a linear gradient of n-butanol from 0.06 to 0.275% in the same buffer over 170 min and detected by their fluorescence at the excitation wavelength of 320 nm and the emission wavelength of 400 nm. The structures of the N-linked sugar chain corresponding to various major peaks was determined by 500 MHz 1H-NMR using preparations obtained from 20 mg of hEPO. 1, an epitope of peak 9 found during the purification sequence; 2, triantennary saccharide; 3 and 4, triantennary saccharides with one N-acetylactosaminyl repeat; 5, biantennary saccharide; 6, triantennary saccharide; 7, tetraantennary triantennary saccharide; 8, tetraantennary triantennary saccharide with two N-acetylactosaminyl repeats; 11, triantennary saccharide with three N-acetylactosaminyl repeats. a, whole; b, N24; c, NS3; d, NS3.

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Fig. 6. CFU-E colony-forming activity of the deglycosylated forms of hEPO. Bone marrow cells (1 × 10⁶) obtained from C57Bl/6 mice (3-days-pregnant) were plated into 35-mm dishes in 1.0 ml of α-luminal minimal medium containing 0.9% methylcellulose, 30% fetal calf serum, 1% bovine serum albumin and 0.1 mM 2-mercaptoethanol, with various concentrations of the intact and the deglycosylated forms of hEPO. The cultures were incubated for two days at 37°C in a humidified 5% CO₂ atmosphere. Colonies containing more than eight cells were scored using an inverted microscope. The assay was carried out in duplicate for each point. O, intact hEPO; ●, NG(2)-hEPO; □, NG(1)-hEPO; ▲, NG(0)-hEPO.

Fig. 7. Binding of the deglycosylated forms of hEPO to target cells. PMEL (5 × 10⁶) obtained from C57Bl/6 mice (3-days-pregnant) were incubated with 0.1 mM [125I]hEPO and the various deglycosylated hEPO derivatives (0.024-6.25 nM) as competitors at 19°C for 3 h in 100 μl of the binding medium. The incubation mixture was then centrifuged over 200 μl of 10% sucrose solution in polyethylene tubes and centrifuged for 1 min at 4°C and 5000 × g. The tubes were cut off just above the cell layer after freezing at -80°C, and cell-associated radioactivity was counted in an Aloka gamma counter. A, intact hEPO; ●, NG(2)-hEPO; □, NG(1)-hEPO; ▲, NG(0)-hEPO.
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**Table I. Amino Acid Composition of the deglycosylated forms of hEPO**

|      | NEPO | Intact | NG(0) | NG(1) | NG(2) | OG(0) |
|------|------|--------|-------|-------|-------|-------|
|      | mol/mol protein |      |       |       |       |       |
| Asx  | 12.1 | 12.5   | 12.4  | 12.2  | 12.5  | 12    |
| Glx  | 18.2 | 19.0   | 18.8  | 18.6  | 18.9  | 19    |
| Ser  | 9.4  | 9.7    | 9.8   | 9.6   | 9.6   | 10    |
| Gly  | 10.2 | 9.8    | 9.7   | 9.7   | 9.9   | 9     |
| His  | 1.9  | 2.1    | 2.1   | 2.1   | 2.0   | 2     |
| Arg  | 10.6 | 11.6   | 11.5  | 11.3  | 11.9  | 12    |
| Thr  | 10.9 | 11.1   | 11.0  | 11.1  | 11.0  | 11    |
| Ala  | 19.0 | 19.0   | 19.0  | 19.0  | 19.0  | 19    |
| Pro  | 8.3  | 8.0    | 8.3   | 8.3   | 8.1   | 8     |
| Tyr  | 3.6  | 3.6    | 3.7   | 3.6   | 3.6   | 4     |
| Val  | 10.8 | 10.6   | 10.8  | 10.6  | 10.6  | 11    |
| Met  | 0.9  | 0.8    | 0.8   | 0.8   | 0.8   | 1     |
| Trp  | n.d. | n.d.   | n.d.  | n.d.  | n.d.  | n.d.  |
| Lys  | 6.7  | 7.2    | 7.0   | 6.9   | 7.2   | 8     |

|      | Theoretical Value* |
|------|--------------------|
| Asx  | 12.5               |
| Glx  | 12.5               |
| Ser  | 9.6                |
| Gly  | 9.6                |
| His  | 2.0                |
| Arg  | 12.0               |
| Thr  | 11.0               |
| Ala  | 19.0               |
| Pro  | 8.3                |
| Tyr  | 3.6                |
| Val  | 10.6               |
| Met  | 0.8                |
| Trp  | n.d.               |
| Lys  | 7.2                |

a. Values from the amino acid sequence.
b. n.d., not determined.

c. Not determined.

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**Table II. Sugar Composition of the deglycosylated forms of hEPO**

|      | NEPO | Intact | NG(0) | NG(1) | NG(2) | OG(0) |
|------|------|--------|-------|-------|-------|-------|
| Fucose| 3.6  | 2.3    | 1.7   | 1.7   | n.d.  |
| Mannose| 8.5  | 6.4    | 3.2   | 3.2   | n.d.  |
| Galactose| 12.7 | 10.2   | 5.6   | 5.6   | 1.1   |
| Galactosamine| 1.0  | 1.3    | 1.3   | 1.3   | 0.03  |
| Glucosamine| 16.7 | 13.0   | 4.9   | 4.9   | 0.3   |
| Sialic acid| 11.2 | 8.3    | 5.1   | 5.1   | 1.7   |

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**Table III. Site-specific N-glycosylation of hEPO**

|      | NEPO | N24 | N38 | N83 | N24 + N38 + N83 |
|------|------|-----|-----|-----|-----------------|
| Type of N-linked sugar chains |      | N24 | N38 | N83 | Whole           |
| Biantennary | 16.5 | 0.1 | n.d.| 5.5 | 4.9             |
| Trisaccharide | 26.7 | 5.4 | 10.4| 14.2| 14.6            |
| Tetrasaccharide | 35.3 | 55.3| 56.8| 49.1| 49.4            |
| Tetra + Lac 1 | 17.0 | 30.3| 25.8| 24.4| 23.7            |
| Tetra + Lac 2 | 4.3  | 8.3 | 8.1 | 6.2 | 6.6             |
| Tetra + Lac 3 | 0.3  | 0.5 | 1.0 | 0.6 | 0.7             |

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a. Biantennary saccharide with one N-acetylactosaminyl repeat
b. Trisaccharide with two N-acetylactosaminyl repeats
c. Tetrasaccharide with three N-acetylactosaminyl repeats

d. Not detected

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**Fig. 8. Relationship between the number of sialic acids and the biological characteristics.** The half-maximal doses in CFU-E colony-formation expressed as ED50 and the Kd values of the intact and the de-N-glycosylated forms of hEPO shown in Table IV were plotted against the number of sialic acids bound to the hEPO derivatives.