Isolation and Characterization of Conformation Sensitive Antierythropoietin
Monoclonal Antibodies: Effect of Disulfide Bonds and Carbohydrate on
Recombinant Human Erythropoietin Structure

By Steve Elliott, David Chang, Evelyne Delorme, Christine Dunn, Joan Egrie, James Giffin, Tony Lorenzini, Caren Talbot, and Lyndal Hesterberg

We have isolated and characterized three anti-recombinant
human erythropoietin (rHuEPO) monoclonal antibodies (MoAbs) that recognize nonoverlapping epitopes on
rHuEPO. Anti-EPO MoAb D11 neutralizes rHuEPO activity whereas MoAbs F12 and 9G8A do not. This suggests that
D11 may bind to the rHuEPO active site. MoAbs F12 and D11 recognize conformation dependent epitopes whereas 9G8A
does not. Immunoassays were developed for each mono-
clonal. The 9G8A immunoassay was novel and useful be-
cause immunoreactivity increased when rHuEPO was dena-
tured. Disruption of disulfide bonds or removal of carbohy-
drate increased 9G8A immunoreactivity, which suggests that
these elements are important for rHuEPO structure or
stability.

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MATERIALS AND METHODS

Materials. Purified monkey EPO (rmkyEPO), glycosylated
rHuEPO from Chinese hamster ovary (CHO) cells, and unglyco-
sylated rHuEPO expressed in Escherichia coli were obtained from T.
Strickland and Ken Aoki (Amgen, Thousand Oaks, CA). Unglyco-
sylated rHuEPO expressed in E coli was purified and refolded as
described previously.23 The E coli expressed rHuEPO has assumed
the proper protein fold as assessed by circular dicroism and fluores-
cence spectroscopy. In addition, in vitro EPO bioassays revealed
an approximate twofold to threefold increase in activity relative to
rHuEPO produced in CHO cells. Mouse EPO (MsEPO) was obtained
from Boehringer Mannheim (Indianapolis, IN). Recombinant mouse
EPO (rMsEPO) was produced from CHO cells (obtained from G.
Goldwasser, University of Chicago) and was obtained from T.
Strickland and K. Aoki (Amgen). Horseradish peroxidase (HRP)
labeled proteins were prepared by the method of Tijssen.25 The EPO
standard used in assays was purified rHuEPO.23

To prepare denatured rHuEPO, it was treated with 5.6 mol/L
GnHCl, 40 mmol/L diethanol, 50 mmol/L Tris pH 8.5 for 60
minutes at room temperature (RT) then stored at 4°C and assayed
directly (preparation 1). A second denatured preparation was made
in the same way except 0.1 vol of 1.5 mol/L iodoacetic acid sodium
salt was subsequently added to prevent reformation of disulfide
bonds. Incubation was for 20 minutes at RT. The denatured rHuEPO
solution was then extensively dialyzed against phosphate buffered
saline (PBS; 10 mmol/L Na2PO4 pH 6.8/150 mmol/L NaCl). The
protein concentration for denatured preparation 1 was determined
from the absorbance at 280 nm of the original rHuEPO and then
was corrected for dilutions. The protein concentration of preparation
2 was determined by Bradford protein assay (BioRad, Richmond,
CA).

For purified proteins, specific activities were calculated from rela-
tive activity by dividing by protein concentration calculated from
the absorbance at 280nm or Bradford protein assay. In all other
cases, immunoreactivities relative to a rHuEPO standard are reported
in units per milliliter (U/mL).

Anti-rHuEPO polyclonal antibodies were raised in rabbits against
purified rHuEPO produced in CHO cells. Platelet-derived growth

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factor (PDGF) MoAb (162) was obtained from Margery Nicolson (Amgen).

Hybridoma/MoAb production. Balb c mice were immunized with rHuEPO (Amgen) using Freund's adjuvant. Hybridoma lines were made using the polyethylene glycol (PEG) procedure as described.\(^{24}\) Cell lines were cloned from single cells following limiting dilution.

Ascites fluid was obtained from Balb cXCD-1 hybrid mice.\(^{25}\) MoAbs were purified by ammonium sulfate precipitation (F12), with protein A (D11, 9G8A), or by hydroxyapatite column chromatography (D11) as described.\(^{26,27}\) Purified MoAbs were aliquoted and stored at less than 10\(^\circ\)C.

Western immunoblotting. Samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to standard methods. Proteins were transferred to 0.2 μm nitrocellulose (Schleicher and Schuell, Keene, NH) using a transblot apparatus (Idea Scientific, Corvallis, OR). The blots were blocked for 1 hour with PBS buffer containing 10% goat serum and antibody in PBS buffer with 5% goat serum and allowed to incubate overnight.

The blot was washed with PBS and then processed with a Vectastain antitumor IgG kit as described by the manufacturer (Vector Laboratories, Burlingame, CA) and developed with 4-CN (GIBCO-BRL, Grand Island, NY).

Radioimmunoassay (RIA). RIA-P uses an anti-rHuEPO polyclonal antibody raised in rabbits and has been described previously.\(^{23,24}\) RIA-N\(^{6}\) uses a rabbit polyclonal antibody against a synthetic peptide (amino acids 1 to 20 of human EPO).

For RIA assays with 9G8A, purified rabbit anti-rHuEPO polyclonal antibody (862) were coated on polystyrene wells at RT for 2 hours in 0.05 mol/L carbonate/bicarbonate pH 9.2 buffer. The wells were then blocked at RT for 30 minutes with 5% bovine serum albumin (BSA) in PBS. Standards or controls (50 μL) in PBS with 1% BSA were incubated in the wells for 3 hours at RT. Excess purified 9G8A antibody (50 μL of 30 μg/mL) was then added and incubated overnight at RT. The next day the wells were washed 3 times with KPL wash solution (Kirkegard and Perry Labs Inc, Gaithersburg, MD), then incubated at RT for 45 minutes with \(^{125}\)I-rabbit antitumor IgG (New England Nuclear, Boston, MA) diluted in normal horse serum (200,000 cpm per well). The wells were then washed 5 times with KPL wash solution and counted.

Enzyme-linked immunosorbent assay (ELISA). To prepare rHuEPO coated wells, 150 ng of rHuEPO in 50 ml/mole Tris, 150 mmol/L NaCl, 10 mmol/L EDTA pH 7.4 was coated onto Dynatech (Chantilly, VA) round bottom microtiter wells during an overnight incubation. The wells were then blocked overnight to prevent non-specific binding. The blocked plates were dried for up to 72 hours and stored at 2 to 8°C until use.

To prepare anti-Fc wells, a 2 μg/mL solution of goat-antimouse (Fc) antibody (Kirkegard and Perry Labs Inc) was incubated overnight in round bottom microtiter wells for an overnight incubation. The wells were then blocked overnight to prevent non-specific binding. The blocked plates were dried for up to 72 hours and stored at 2 to 8°C until use.

Microtiter wells coated with the respective MoAb were made using the methods outlined by Tijssen.\(^{29}\) Coated wells were stored at 2 to 8°C until use.

Several methods were used to determine the immunoreactivity of MoAb solutions or to determine rHuEPO concentrations. All reactions were done in microtiter plates with either TMB or OPD (Kirkegard and Perry Labs Inc) as substrates. Reactions with TMB were stopped with 0.5 mol/L H₂SO₄ whereas OD reactions were stopped with 2.5 mol/L H₂SO₄. The plates were read at 450 nm/630 nm for TMB and at 490 nm/630 nm for OPD.

(1) Anti-EPO antibody containing samples were diluted 1:1 in buffer and added to rHuEPO coated wells and incubated 2 hours at 37°C. The plate contents were removed and rabbit antimouse HRP conjugated secondary antibody was added and incubated at 37°C for 30 minutes. The plates were then washed 5 times with TEN buffer (50 mmol/L Tris pH 7.4, 10 mmol/L EDTA, 150 mmol/L NaCl) and then developed.

(2) Anti-EPO antibody containing samples were incubated for 2 hours at RT in antimouse Fc wells. After removal of the solution, HRP conjugated rHuEPO was added to each well and allowed to incubate at 37°C for 2 hours. The wells were then washed 4 times with TEN containing 0.01% Triton X-100 (Bio-Rad, Richmond, CA) and then developed.

(3) Anti-EPO antibody containing samples were incubated in the antimouse (Fc) wells for 2 hours at RT. After removal of the solution, EPO containing solutions were added and allowed to incubate at RT for 2 hours. The solution was removed and 50 μL HRP conjugated anti-rHuEPO rabbit antibody was added and incubated at 37°C for 2 hours. The wells were then washed 4 times with 0.1% Triton X-100 in TEN and developed.

(4) EPO containing samples were added to antibody coated wells and incubated at RT for 2 hours. The solution was removed and HRP conjugated anti-rHuEPO polyclonal antibody, raised in either rabbits (R) or goats (G), was then added to each well and incubated at 37°C for 2 hours. The wells were then washed 4 times with 0.1% Triton X-100 in TEN and developed.

Antibody competition assays. Microtiter wells were coated for 2 hours with MoAbs 9G8A, F12, or D11 in 0.05 mol/L carbonate/bicarbonate pH 9.2 buffer. To test for competition, antibodies, and rHuEPO (25,000 cpm in 100 μL; Amersham, Arlington Heights, IL) were added to the coated wells and incubated at RT for 3 hours. The wells were then washed 5 times with KPL wash solution and counted in a gamma counter.

In vitro bioassays. Erythroid colony forming assays were performed as described by Iscove et al with the following modifications. The mononucleated cells from human bone marrow cells were partially purified on a ficoll-hypaque cushion and washed in Iscove medium before plating on tissue culture dishes to remove adherent cells. The culture medium contained 0.9% methylcellulose instead of 0.8%, and did not include any BSA. The erythroid colonies were scored after 7 to 10 days of culture.

In vitro bioactivity was also determined by measuring thymidine uptake in a factor-dependent cell line, 32D\(^{22,23}\) that had been made dependent on EPO for growth by transfection of a murine EPO receptor gene (32D + EPOR\(^6\)). Assays were performed as described using rHuEPO as standard with the following modifications. 32D cells were factor-starved for 3 to 5 hours in assay medium lacking EPO. Test samples or EPO standard (rHuEPO) were added to wells in a 96-well microtiter plate. Fifty microliters of starved cells was then added (15,000 cells/well) and plates were incubated in a humidified incubator at 37°C and 6% CO₂. After 18 to 24 hours, 50 mL methyl-3-H-thymidine (1 μCi/mL; 20 Ci/mmol) diluted 1:100 in assay medium was added. Plates were incubated for an additional 4 hours at 37°C and 6% CO₂. Labeled cells were then procured onto glass fiber filters using a PHD cell harvester (Cambridge Technology Inc, Watertown, MA) and deionized water as a washing solution followed by 2-Propanol. The filters were then dried and counted.

EPO neutralization assays. To determine whether antibodies would neutralize rHuEPO bioactivity, rHuEPO standard was incubated overnight with various amounts of antibodies in PBS buffer at RT. The amount of rHuEPO standard was 300 mUnits. This amount was chosen because 300 mUnits gave a response that was in the middle of the standard curve. The next day the samples were assayed with the erythroid colony formation in vitro bioassay as previously described above.

Construction of rHuEPO variants and transfection of COS-1 cells.
Fig 1. Antibody competition experiments. The indicated amounts of antibodies were mixed with \(^{125}I\) rHuEPO and added to 9G8A, D11, or F12 antibody coated wells then incubated, washed, and counted as described in Materials and Methods. The amount of rHuEPO bound to microtiter with \(^{125}I\) rHuEPO control samples lacking antibody was 75, 3,600, and 1,840 cpm for 9G8A, D11, and F12 wells, respectively.

All rHuEPO variants were constructed by in vitro mutagenesis and supernatants containing these variants were recovered from transfected COS-1 cells as described in the accompanying paper.34

RESULTS

Isolation of MoAbs and initial characterization. Two separate immunizations of mice with the same lot of rHuEPO were done and hybridomas were made. To screen the monoclonals for rHuEPO reactivity, supernatants from 1,300 growth positive wells were screened using anti-Fc wells (experiment one) and supernatants from 1,900 wells were screened with rHuEPO coated wells (experiment two). Twenty clones were chosen for ascites production from the results of experiment 1 and 1 clone was chosen from experiment 2. From these clones three different MoAbs were chosen for additional study. Monoclonals F12 and D11 were recovered from experiment 1 and 9G8A was recovered from experiment 2.

Antibody competition experiments. MoAbs F12, D11, and 9G8A were tested for their ability to compete with each other for rHuEPO binding as described in Materials and Methods. We found that all three antibodies would bind rHuEPO in this format. However, the amount of rHuEPO bound by 9G8A was significantly less than by D11 or F12 (40-fold difference). As expected, each of the monoclonals competed with themselves (Fig 1). However, rHuEPO binding by immobilized 9G8A was not inhibited by D11 or F12. This indicates that 9G8A binds to a different site on rHuEPO than either D11 or F12. In a similar manner rHuEPO binding by immobilized D11 was not inhibited by 9G8A and F12. This indicates that D11 binds to a different site on rHuEPO than either 9G8A or F12. F12 binding to rHuEPO was inhibited slightly by D11 and 9G8A. However, the binding could not be completely inhibited and the inhibition was modest compared to inhibition of F12 by itself. These results suggest that the weak inhibition of EPO binding to F12 by D11 and 9G8A is because of an indirect mechanism. This suggests that the three antibodies recognize separate nonoverlapping epitopes.

Fig 2. Neutralization of rHuEPO bioactivity. The indicated quantities of antibodies were incubated with 300 mUnits of rHuEPO then assayed in an in vitro bioassay as described in Materials and Methods. Assay volumes were 1 mL. Percent activity is the activity remaining in the samples relative to a control rHuEPO sample incubated without any antibody. Incubation was with anti-rHuEPO rabbit polyclonal antibody 862, anti-PDGF MoAb 162, anti-rHuEPO MoAb D11, Anti-rHuEPO MoAb 9G8A, and anti-rHuEPO MoAb F12.
Characterization of Anti-Epo MoAbs

Table 1. Immunoreactivity of rHuEPO and EPO Variants

<table>
<thead>
<tr>
<th>Sample</th>
<th>RIA-N U/mg</th>
<th>RIA-P U/mg</th>
<th>F12-R U/mg</th>
<th>D11-R U/mg</th>
<th>9G8A U/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>rHuEPO</td>
<td>204,000</td>
<td>196,000</td>
<td>230,000</td>
<td>230,000</td>
<td>147,000</td>
</tr>
<tr>
<td>rHuEPO (Denatured)</td>
<td>NP*</td>
<td>76</td>
<td>76</td>
<td>15,600</td>
<td>9,800,000</td>
</tr>
<tr>
<td>rHuEPO (E. coli)</td>
<td>180,000</td>
<td>270,000</td>
<td>260,000</td>
<td>74,400</td>
<td>890,000</td>
</tr>
<tr>
<td>rHuEPO (Neuraminidase)</td>
<td>165,000</td>
<td>190,000</td>
<td>176,000</td>
<td>194,000</td>
<td>298,000</td>
</tr>
<tr>
<td>rMkyEPO (Monkey)</td>
<td>565</td>
<td>88,300</td>
<td>&lt;0.6</td>
<td>51,700</td>
<td>68,400</td>
</tr>
<tr>
<td>rMsEPO (Mouse)</td>
<td>356,000</td>
<td>100,600</td>
<td>43</td>
<td>1,700</td>
<td>715</td>
</tr>
</tbody>
</table>

Neutralization of rHuEPO by MoAbs. To determine whether any of the three rHuEPO MoAbs could neutralize rHuEPO activity, rHuEPO was incubated overnight with varying amounts of antibody then in vitro bioactivity (erythropoiesis assay) was determined as described in Materials and Methods. Two control antibodies were also tested, an anti-rHuEPO rabbit polyclonal antibody (862) that had been previously identified as having EPO neutralizing activity and a MoAb directed against human PDGF. The latter antibody would not be expected to neutralize rHuEPO activity. As expected, the polyclonal antibody (862) was very effective at neutralizing rHuEPO activity and the anti-PDGF MoAb had no effect (Fig 2), even at a 6,000-fold excess (10 μg MoAb/300 mUnits rHuEPO). Recovery of anti-EPO antibody in the 862 antiserum by EPO affinity purification was about 0.1 μg antibody per mL. Using this value as an estimate of the anti-EPO antibody concentration in 862 serum, we calculate that 10 ng would neutralize 300 mUnits of rHuEPO (sixfold excess). The ability of F12, D11, and 9G8A to neutralize 300 mUnits of rHuEPO was also tested. F12 and 9G8A had no effect on rHuEPO activity even at a 50-fold excess of antibody. However, D11 antibody was required for 80% inhibition (250-fold excess). Thus, D11 appears to neutralize rHuEPO activity but requires approximately 40-fold more antibody than the 862 polyclonal antibody.

Development of Immunoassays. Initial tests indicated that rHuEPO could be captured by D11 and F12 when the antibodies were immobilized on microtiter plates. Therefore ELISAs were developed for F12 and D11 that involved initial capture of rHuEPO by the monoclonals followed by a second incubation with HRP conjugated anti-rHuEPO polyclonal signal antibodies. Two different secondary signal polyclonals were tested including goat and rabbit derived polyclonal antibodies. The assays with EIA/F12-R (rabbit), and EIA/D11-R (rabbit) gave the same results as that of the previously described RIA-P24,28 for purified rHuEPO (Table 1). The same results were obtained for conditioned medium from rHuEPO transfected COS-1 cells (Table 2). Table 2 also shows that D11 and F12 ELISA assays give the same results when either goat or rabbit secondary antibodies were used. Thus any of the combinations of monoclonals and secondary antibodies described in this report can be used to determine rHuEPO concentration.

Immunoassays using the 9G8A antibody required a different format from D11 and F12 ELISA immunoassays. 9G8A attached to microtiter plates bound rHuEPO poorly (Fig 1). To circumvent this problem a sandwich RIA was developed that involved initial capture of rHuEPO in solution by a rabbit polyclonal antibody (862) attached to a microtiter plate and then a second incubation with 9G8A followed by a third incubation with 125I labeled goat antimouse antibodies. The amount of 125I bound was found to be proportional to the amount of rHuEPO in solution.

Analysis of antibody binding by Western blots. One method to distinguish between conformational and linear epitopes is to see if the antibodies recognize denatured rHuEPO in a Western blot format. In this method, antibodies that recognize linear epitopes would be expected to recognize denatured rHuEPO and antibodies that recognize conformational epitopes would not. Therefore, we performed Western blots using two different amounts of rHuEPO that had been denatured by boiling in SDS and 2-mercaptoethanol. Initial experiments with 1 μg D11 or F12 gave no signal to rHuEPO. Therefore an experiment was performed using 10 μg D11 or F12. As shown in Fig 3, F12 gave no signal in Western immunoblots even with 10 μg antibody. D11 gave a weak signal with 10 μg antibody that increased with increased amount of rHuEPO. However, 9G8A gave a strong signal with 10-fold less antibody (1 μg) than that used for

Table 2. Immunoreactivity of rHuEPO and EPO Variants in Conditioned Medium

<table>
<thead>
<tr>
<th>Sample*</th>
<th>RIA-N U/mL</th>
<th>RIA-P U/mL</th>
<th>F12-R U/mL</th>
<th>D11-R U/mL</th>
<th>9G8A U/mL</th>
<th>In Vitro Activity† U/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>rHuEPO</td>
<td>60</td>
<td>66</td>
<td>82</td>
<td>60</td>
<td>83</td>
<td>71</td>
</tr>
<tr>
<td>Cys7/Ser rHuEPO</td>
<td>12</td>
<td>0.6</td>
<td>0.2</td>
<td>0.6</td>
<td>0.4</td>
<td>11</td>
</tr>
<tr>
<td>Cys33/Ser rHuEPO</td>
<td>25</td>
<td>5</td>
<td>0.3</td>
<td>0.23</td>
<td>10</td>
<td>17</td>
</tr>
<tr>
<td>Cys181/Ser rHuEPO</td>
<td>1.2</td>
<td>&lt;0.3</td>
<td>&lt;0.06</td>
<td>&lt;0.06</td>
<td>&lt;0.06</td>
<td>&lt;0.06</td>
</tr>
</tbody>
</table>

* Conditioned medium was from COS-1 cells expressing the indicated rHuEPO variant.
† In vitro activity was determined by measuring 3H thymidine uptake in 32D + EPOR cells.
F12 or D11. It is unlikely that the differences in immunoreactivity in Western blots between MoAbs F12 and D11 were because of different affinities for the antigen because D11 was as sensitive as F12 in ELISA assays when similar amounts of antibody were used (see Fig 1). It is instead likely that F12 recognizes a conformation dependent epitope and D11 recognizes primarily folded rHuEPO but retains weak immunoreactivity to denatured rHuEPO.

**Immunoreactivity of the antibodies to denatured rHuEPO in RIA and ELISA.** To examine the immunoreactivity of the antibodies in more detail, purified rHuEPO was denatured with dithiothreitol in the presence of GlnHCl and assayed directly. In this case we found that about one third of the biological activity was recovered when the sample was diluted for assay. This indicated that some of the rHuEPO had refolded into an active conformation. However, when reformation of disulfide bonds was prevented by subsequent treatment with iodoacetate rHuEPO, the biological activity was undetectable (<2 U/mg). Thus blocking disulfide bond formation prevented reformation into an active conformation.

The immunoreactivity of the denatured-iodoacetate treated rHuEPO was assayed (Table 1). F12 immunoreactivity determined by EIA/F12-R was reduced approximately 3,000-fold relative to rHuEPO (Table 1). Immunoreactivity determined with D11-R was reduced 15-fold. RIA-P also showed reduced immunoreactivity to denatured rHuEPO (250-fold). These results support the proposal that F12 and D11 and the polyclonal antibody used in RIA-P recognize primarily folded rHuEPO and that F12 immunoreactivity is more dependent on proper rHuEPO conformation than D11.

We have observed that 9G8A immunoreactivity increased when rHuEPO containing samples had been frozen and thawed. This suggested that unfolding of rHuEPO increased 9G8A antibody binding. To test this possibility, the denatured and iodoacetate treated rHuEPO was assayed using RIA-9G8A. We found a 65-fold increase in 9G8A immunoreactivity with this sample as compared to untreated rHuEPO (Table 1). The denatured EPO sample that had not been iodoacetate treated showed a 30- to 40-fold increase in immunoreactivity as well (data not shown). This result along with the observations that 9G8A works well in Western blots and that 9G8A binds correctly folded rHuEPO in solution poorly suggests that 9G8A recognizes a linear epitope and that the epitope is either buried and exposed on denaturation or in a different conformation in folded versus denatured rHuEPO.

RIA-N uses an antibody raised to a synthetic human EPO peptide, amino acids 1 to 20. Many antipeptide antibodies are tolerant to changes in protein conformation. To test this...
posibility the denatured rHuEPO sample was assayed with RIA-N. In this case the antibody reacted with the denatured rHuEPO sample. However, the slope of the linear range of the curve was slightly different from that of the rHuEPO standard. We estimated the relative immunoreactivities of rHuEPO and denatured rHuEPO measured by RIA-N by calculating the midpoint of the dose response curves. The midpoint for the denatured sample was 69% that of the rHuEPO standard. We used the standard curve to estimate the denatured rHuEPO concentration using points at the top and bottom of the linear range of curves from three different experiments. The highest value obtained was 255,000 U/mg and the lowest was 70,000 U/mg. This range compares favorably with the specific immunoreactivity to rHuEPO (204,000 U/mg, Table 1). These results suggest that the immunoreactivities measured by RIA-N are similar but there are some differences in how the antibodies contact rHuEPO versus denatured rHuEPO.

Effect of disruption of disulfide bonds on immunoreactivity. Recombinant human EPO contains two disulfide bonds that join Cys7 to Cys16' and Cys33 to Cys33.33 To determine the effect of disruption of these disulfide bonds on folding and activity, Cys to Ser mutations were introduced into rHuEPO and the variants were expressed in COS-1 cells. The concentration of each rHuEPO variant in the supernatants was determined by RIA-N and other immunoassays. There was a 20- to 30-fold increase in measurements by RIA-9G8A relative to RIA-N when either disulfide bond was disrupted. These Cys to Ser rHuEPO variants were also not detected well by RIA-P, EIA/D11-R, or EIA/F12-R as compared to RIA-N. Unlike EIA/D11-R, immunoreactivity measured by EIA/D11-G appeared to be less affected by the mutations. Estimates of the concentrations of Cys7'/Ser rHuEPO and Cys33'/Ser rHuEPO matched closely the values obtained by RIA-N (Table 2). This suggests that goat and rabbit secondary antibodies used in immunoassays differ in their immunoreactivities to rHuEPO variants with disrupted disulfide bonds. All the immunoassays had similar results with natural sequence rHuEPO. These results suggest that the conformation of rHuEPO is altered when either disulfide bond is disrupted.

EIA/D11-R immunoassays revealed additional information about the effect of mutations in the disulfide bonds on folding. D11-R immunoreactivity was reduced approximately 30-fold (relative to RIA-N) for the Cys7'/Ser mutation, at least 20-fold for Cys33'/Ser mutation but only 2.5 fold for the Cys7'/Ser mutation. Neither Cys7' nor Cys33' appear to be part of the D11 epitope.44 RIA-P results were qualitatively similar to that of EIA/D11-R. That is, the Cys7'/Ser mutation had a greater effect on this immunoassay than did the Cys33'/ Ser mutation. These results suggest that disruption of either disulfide bond in rHuEPO reduced the immunoreactivity measured by conformation sensitive antibodies, presumably because of a change in tertiary structure, and that the Cys7'/Cys16' disulfide bond is more important for structure than the Cys7'/Cys33' bond.

We wished to determine what role the disulfide bonds play in in vitro biological activity. The supernatants containing rHuEPO and the rHuEPO variants were therefore assayed for their ability to stimulate thymidine uptake in the factor dependent cell line 32D+EPO. The results are shown in Table 2. rHuEPO expressed in COS cells had an in vitro biological activity that was comparable to the concentrations determined by RIA and EIA immunoassays. This suggests that the rHuEPO made under these conditions is fully active. In contrast there was no biological activity detected for either the Cys7'/Ser or Cys16'/Ser variants. The biological activity relative to RIA-N immunoassay was reduced over 100-fold for Cys16'/Ser and over 1,000-fold for Cys7'/Ser variants. Unlike variants with mutations in the Cys7-Cys16' disulfide bond, there was biological activity detected for a Cys7'/Ser rHuEPO variant. The in vitro activity was reduced approximately six fold (relative to RIA-N and EIA/D11-G). However, the biological activity matched closely the EPO concentration determined by RIA-P, 5 U/mL versus 4 U/mL. These results suggest that both disulfide bonds are important for in vitro biological activity but the Cys7'-Cys16' disulfide bond is less important and that RIA-P measurements may underestimate the true concentrations for these rHuEPO variants.

Immunoreactivity of antibodies to unglycosylated rHuEPO. Human EPO contains 3 N-linked and 1 O-linked carbohydrate chains. To see if the carbohydrate affected antibody binding, rHuEPO was expressed in E. coli to produce material lacking carbohydrate. The rHuEPO was purified and refolded into an active conformation as evidenced by in vitro bioassays and circular dichroism.25 Both EIA/F12 and RIA-P gave values in immunoassay that compared favorably with each other with unglycosylated rHuEPO (Table 1). The specific immunoreactivities of unglycosylated rHuEPO measured by EIA/F12 and RIA-P were slightly higher than that of glycosylated rHuEPO. This result indicates that carbohydrate is not required for RIA-P and EIA/F12 immunoreactivity and may slightly inhibit immunoreactivity. Immunoreactivity measured by RIA-N appeared to be the same for both glycosylated and unglycosylated rHuEPO. D11 immunoreactivity appeared to be reduced to unglycosylated rHuEPO. Apparent immunoreactivity with 9G8A was approximately 40-fold higher for unglycosylated rHuEPO than for glycosylated rHuEPO. A similar result was found for rHuEPO treated with N-glycanase to remove N-linked carbohydrate (data not shown). Therefore, either carbohydrate 9G8A binding, unglycosylated rHuEPO is less stable resulting in changes in folding that affect D11 and 9G8A immunoreactivity, or it has a more exposed 9G8A epitope.

To examine the role of carbohydrate on immunoreactivity in more detail, sialic acid on glycosylated rHuEPO was removed by neuraminidase treatment to see if sialic acid affected immunoreactivity. All the antibodies appeared to react similarly to asialo rHuEPO except 9G8A whose immunoreactivity was increased two fold. We have found that the immunoreactivity measured by RIA-9G8A to asialo rHuEPO changed with age of the sample. Fresh samples had much lower immunoreactivity than samples that had been frozen and thawed several times.

Antibody immunoreactivity of nonhuman EPO. Examination of immunoreactivity to molecules derived from different species can provide information about antibody epitopes. In
addition, we wished to determine which immunoassays could be used to measure EPO titers in monkeys and mice. Therefore, we examined the immunoreactivity of the 3 MoAbs and 2 polyclonal antibodies to monkey and mouse EPO. Monkey EPO differs from human EPO at 14% and mouse EPO at 31,32,33 of the 165 positions found in rHuEPO. In addition the amino terminus in monkey EPO is extended by 3 amino acids relative to human EPO.34 It has been shown previously that RIA-P provides an accurate measure of monkey EPO concentration35 and that RIA-N does not because of differences in the amino terminus.36 Immunoreactivity measured by EIA/DI1-R and RIA-9G8A of monkey EPO compared favorably to that measured by RIA-P. This suggests that the amino acid differences between monkey and human EPO are not at positions recognized by 9G8A, DI1 or the antibody used in RIA-P. F12 immunoreactivity to monkey EPO was reduced over 10,000-fold (EIA/F12-R, Table 1). This indicates that the amino acid differences between human EPO and monkey EPO are part of or affect the F12 epitope. Immunoreactivities measured by RIA-P, EIA/F12-R, EIA/DI1-R, and RIA-9G8A to RIA-N were all reduced when rMsEPO was assayed (3, 8,000, 200, and 500-fold, respectively). Similar results were obtained with mouse EPO obtained from Boehringer Mannheim (data not shown). These results suggest that the amino acid differences in mouse EPO may include antibody epitopes for all the monoclonals. Another possibility is that rMsEPO is folded differently than is rHuEPO and thus the epitopes are altered.

**DISCUSSION**

Three different monoclonals and two polyclonals raised against rHuEPO were isolated and characterized. Immunoassays with each have been developed and all can be used to determine concentrations of rHuEPO. Competition experiments indicate that the three MoAbs recognize nonoverlapping epitopes. In vitro EPO bioassays indicate that DI1 is neutralizing whereas F12 and 9G8A are not. This suggests that the DI1 epitope is part of, or overlaps, the receptor binding site and F12 and 9G8A do not. However, the results with 9G8A must be viewed with caution. The affinity of 9G8A for rHuEPO in solution is low and therefore the antibody may not effectively block receptor binding and be unable to neutralize.

The antibodies and immunoassays described here can be used to determine whether EPO conformation has been altered. The antibody used in RIA-N appears to be relatively tolerant to changes in conformation as evidenced by its ability to react with denatured rHuEPO. In contrast, the monoclonals DI1 and F12 and the rabbit anti-rHuEPO polyclonal antibody used in RIA-P appear to recognize conformational epitopes. Thus, their immunoreactivities are reduced for denatured rHuEPO. F12 is much more sensitive to changes in rHuEPO conformation than DI1. In addition we describe a novel immunoassay based on the MoAb 9G8A. Immunoreactivity with this antibody increases when the protein is denatured.

The utility of using such conformation sensitive immunoassays is illustrated in experiments used to establish the role of rHuEPO disulfide bonds on structure and activity. We have found that Cys33 to Ser, Cys7 to Ser and Cys84 to Ser mutations all result in an increase in immunoreactivity with 9G8A and decreases in immunoreactivity measured by RIA-P, EIA/F12, and EIA/DI1-R relative to RIA-N. A similar result was found for a Cys33 to Pro mutation.33 Thus these mutations appear to disrupt rHuEPO structure and presumably affect immunoreactivity of RIA-P and F12 indirectly. These mutations also affect in vitro biological activity. Mutations that disrupt the Cys25-Cys26 disulfide bond appear to alter rHuEPO structure and in vitro biological activity to a greater degree than mutations that disrupt the Cys29-Cys31 disulfide bond. This is evidenced by the greater effect of the mutations on DI1, RIA-P immunoreactivity, and in vitro bioactivity. Therefore, both of the disulfide bonds play an essential role in maintaining the protein in the proper folded state which in turn is required for full biological activity.

The importance of the Cys25-Cys31 bond in biological activity is in agreement with that of Boissel et al.30 However there are apparent differences on the role of the Cys29-Cys31 disulfide bond. It was reported that mutation of either Cys29, Cys31 or both to Tyr resulted in normal in vitro biological activity.40 Several explanations for the difference are apparent. One possibility is that Tyr substitutions are more favorable substitutions for rHuEPO conformation than the Ser or Pro substitutions we introduced. The EPO variants were not purified in either study and the quantitation of EPO concentration depended on immunoassay. Thus another possibility is that the different results are because of disparities in the method used to quantitate EPO concentration. We have prepared a similar rabbit polyclonal antibody (used in RIA-P) to that used by Boissel et al, and found that the EPO concentration determined by RIA-P matches the in vitro activity. In our calculation of specific activity we used the RIA-N and EIA/DI1-G immunoassays to estimate rHuEPO concentration. These immunoassays give higher estimates of rHuEPO concentration and thus reduced specific activity estimates.

The importance of carbohydrate for rHuEPO structure/stability was suggested by immunosassay. 9G8A immunoreactivity increased when carbohydrate was removed. This result suggests several possibilities. The epitope recognized by 9G8A may be masked by the carbohydrate. This appears unlikely because removal of any single N-linked oligosaccharide chain by in vitro mutagenesis only modestly affects 9G8A immunoreactivity,41 and removal of the O-linked site has no effect, but 9G8A immunoreactivity increases dramatically when all 3 N-linked sites are removed. Thus, the effect on 9G8A immunoreactivity is elicited only with completely unglycosylated rHuEPO. A second possibility is that removal of carbohydrate results in a change in rHuEPO structure that exposes the 9G8A epitope. However, a change in structure of rHuEPO when carbohydrate was removed could not be detected by circular dichroism.23 Therefore, the change in rHuEPO structure when carbohydrate is removed would have to be subtle and affect structure to a degree detectable by antibody binding and not by circular dichroism. A third possibility is that unglycosylated rHuEPO is unstable and quickly unfolds. It has been reported that removal of carbo-
hydrate results in a decrease in conformational stability. In addition the material does precipitate with prolonged storage even at 4°C.

Assays on EPO molecules from different species indicate that monkey EPO is recognized by D11 and 9G8A but immunoreactivity is reduced with F12. Mouse EPO is recognized poorly by F12 and partially by D11 and 9G8A. Fine-structure epitope mapping indicates that F12 recognizes residues at positions 31 to 33, 86 to 88, and 138. Mouse EPO differs from rHuEPO at positions 32 to 34 and 88 and monkey EPO differs at position 32 and 88. Thus these differences can account for the reduced F12 immunoreactivity. 9G8A recognizes a linear epitope near the N-terminus. In the N-terminal region at the 9G8A binding site, mouse, rHuEPO, and monkey EPO differ only at amino acid position 16. Mouse EPO has a lle at this position and rHuEPO and monkey EPO have a Leu. Thus, the difference in 9G8A immunoreactivity can be accounted for by the differences at position 16. Epitope mapping data suggests that D11 recognizes amino acids 64, 67, 72, 75, 100, 103, 106, and 110. However, mouse and rHuEPO have identical residues at all of these positions. It is possible that other, unidentified, residues are part of the D11 epitope and those differ in mouse and rHuEPO. Another possibility is that the reduced immunoreactivity is caused by conformational differences in the D11 epitope between mouse and rHuEPO.

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Isolation and characterization of conformation sensitive anti-erythropoietin monoclonal antibodies: effect of disulfide bonds and carbohydrate on recombinant human erythropoietin structure

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