Characterization and Use of Monoclonal Antibodies Directed Against Human Erythropoietin That Recognize Different Antigenic Determinants

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We have established four hybridoma cells that produce monoclonal antibodies (MoAbs) R2, R4, R6, and R12 directed toward recombinant human erythropoietin (rHuEPO). MoAbs R2, R4, and R6 bound to EPO with high affinities (kd = ~2.4, and 1 nmol/L, respectively) but MoAb R12 had a low affinity (240 nmol/L). These antibodies inhibited the biological activity of rHuEPO and EPOs from humans, rats, mice, and rabbits. This inhibition was due to the blocking of EPO binding to the target cells. The fully deglycosylated rHuEPO bound to the MoAbs, indicating that they recognized peptide sequences of the antigen but not the carbohydrates attached to the antigen. An immunosorbent column with the immobilized MoAb R2 was effective for the rapid purification of EPO. MoAb R6 bound to EPO at a site(s) different from those to which other MoAbs bound. Based on this finding, a sensitive and rapid enzyme-linked immunosorbent assay of EPO, in which EPO was sandwiched between two MoAbs (R2 and R6), was developed. The assay measured plasma levels of EPO as low as 5 mU/mL within several hours.

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ERYTHROPOIETIN (EPO) is a sialylglycoprotein that stimulates RBC production by promoting both the growth of late erythroid precursor cells and their maturation into proerythroblasts. The advantages of the use of a monoclonal antibody (MoAb) for immunochemical studies of the antigen have been amply documented. So far, preparation of MoAbs against human EPO has been reported from four laboratories. Weiss et al first described an EPO-directed hybridoma that was obtained by the fusion of rat spleen cells and mouse myeloma. This hybridoma proliferated in the ascitic fluid of nude mice but not in those of normal ones, which is disadvantageous for preparation of large amounts of the MoAb. Sue and Sytkowski described an MoAb directed toward the aminoterminal region of human EPO. Immune complexes formed between this MoAb and EPO had full biological activity, so involvement of the aminoterminal region in receptor binding is unlikely. Wognum et al have prepared a number of MoAbs against human EPO and reported their basic properties such as the affinities of the MoAbs to the antigen and their neutralization of the biological activity of EPO, but their use for immunochemical studies of EPO has not been described. We reported an EPO-directed MoAb that binds to the antigen after it is treated with sodium dodecyl sulfate (SDS). It appears that this antibody binds to the epitope exposed by a conformational change of EPO caused by the SDS. This antibody worked efficiently in the purification of EPO from human urine and human recombinant EPO (rHuEPO) from the supernatant of cultured cells, but treatment of preparations containing EPO with SDS is time consuming and this antibody would not be useful if EPO not treated with SDS.

We did this study to prepare MoAbs capable of binding with the native EPO and thereby to achieve the following purposes: (1) establishment of a more rapid procedure for the purification of EPO by immobilized MoAb; (2) development of a sandwich-type enzyme-linked immunosorbent assay (ELISA), which is easier to handle and more rapid and sensitive than radioimmunoassay (RIA), by use of MoAbs that bind to EPO at different antigenic determinants; and (3) acquisition of an MoAb that binds to EPO at the peptide sequence(s) involved in interaction with its receptor. We established four hybridoma cells that were stable, with rapid growth and high production of antibodies, by the use of rHuEPO as an antigen. One of them was suitable for purification of EPO with a high yield. At least one of these antibodies recognized an epitope(s) different from epitopes recognized by the others. This made it possible to develop a very rapid and sensitive ELISA for EPO. The third purpose is not yet accomplished, but some properties of the antibodies in their interaction with EPO have been analyzed.

MATERIALS AND METHODS

Erythropoietins and their biologic activity. EPO (88,000 U/mg) from the urine of anemic patients and rHuEPO (82,000 U/A 280 nm) from the culture supernatants of BHK cells were purified to homogeneity as described previously. Human plasma that contained EPO at a variety of concentrations were those obtained previously; they were stored at -80°C. The rat, mouse, and rabbit plasma were prepared from animals made anemic by the injection of phenylhydrazine. EPO solutions were diluted with 10 mmol/L phosphate-buffered saline (PBS), pH 7.4, containing 5% bovine serum albumin (BSA) unless otherwise indicated.

EPO was assayed in vitro by use of its stimulatory effect on the incorporation of [3H]-thymidine into DNA in cultured liver cells of fetal mice.

125I-labeled recombinant human erythropoietin. rHuEPO was labeled with 125I by the iodogen method as described previously. The labeled EPO retained full in vitro biological activity and its specific radioactivity was about 100 Ci/gg protein unless otherwise indicated.

Binding of 125I-labeled recombinant human erythropoietin to its receptor. 125I-labeled rHuEPO was bound to mouse erythrocytes and mouse fetal liver cells as described previously with a minor modification. The cell suspension (100 µL containing TSA8 cells or cells from 13-day fetuses of ICR mice was mixed with 50 µL of PBS containing 60 mmol/L HEPES, pH 7.2, 0.3% BSA, 0.6% NaN3, and 125I-labeled EPO (1.8 nmol/L). After incubation for three hours at 15°C, the cells were pelleted, washed once with

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PBS, and suspended in 200 µL of PBS. The suspension was layered on 800 µL of PBS containing 10% BSA and the cells were separated from the unbound ligand by centrifugation. The tube contents were frozen in solid CO₂/ethanol, and the tips were cut off just above the cell pellet to count the radioactivity.

**Deglycosylated erythropoietin.** Deglycosylated rHuEPO was prepared by continuous digestion with neuraminidase, endo-α-N-acetylgalactosaminidase, which removes O-linked carbohydrates attached to proteins, and N-glycanase, which removes N-linked carbohydrates. One hundred micrograms of rHuEPO was incubated with 5 µU of neuraminidase (Seikagaku Kogyo Co, Tokyo) for three hours at 37°C in 250 µL of 100 mmol/L sodium acetate buffer, pH 6.5, containing 10 mmol/L CaCl₂. After incubation of the mixture, the buffer was changed to 25 mmol/L sodium acetate, pH 4.5, by ultrafiltration dialysis with a Centri Cut Mini V-10 (Kurashiki Boseki, Osaka, Japan) and the mixture volume was adjusted to 300 µL. To the mixture, 15 µU of endo-α-N-acetylgalactosaminidase (the gift of Dr Kenji Yamamoto, Kyoto University, Japan)1213 in 10 µL of 0.6 mol/L potassium phosphate, pH 6.0, was added and the mixture was incubated for three hours at 37°C. After digestion, the medium of the mixture was changed to 100 mmol/L sodium phosphate, pH 8.6, containing 20 mmol/L EDTA by ultrafiltration dialysis and the mixture volume was adjusted to 80 µL. To the mixture, 5 U of N-glycanase (Genzyme Corp, Boston) in 20 µL of 50% glycerol containing 2.5 mmol/L NaCl was added and the mixture was incubated for 24 hours at 37°C. Deglycosylated rHuEPO was stored at −20°C until use.

**Cell culture.** Cells were cultured at 37°C in an atmosphere of 5% CO₂/95% air at 100% humidity. All media for cell culture contained 10 U/mL penicillin and 100 µg/mL streptomycin. The Friend erythroleukemia cell line, TSA8, was maintained in RPMI supplemented with 15% of fetal calf serum (FCS; M. A. Bioproducts, Walkersville, MD). Hybridoma cells were maintained in RPMI supplemented with 10% FCS, 2 mmol/L glutamine, and 1 mmol/L pyruvate.

**Preparation and selection of hybridomas.** Female BALB/c mice 8 weeks old were immunized intraperitoneally with 90 µg of pure rHuEPO emulsified 1:1 in Freund’s complete adjuvant in a total volume of 200 µL. Two weeks later, a second immunization was done with 45 µg of the antigen; 2 weeks later, the last immunization was done without the adjuvant. Hybridomas were prepared by procedures described previously.44 Briefly, 1.2 × 10⁶ spleen cells obtained from mice three days after the last immunization were fused with 6 × 10⁵ mouse myeloma cells (P3-NS1/1-Ag4-1). The fused cells were plated in six 96-well tissue culture plates (Nunc, Denmark) and selected in hypoxanthine-aminopterin-thymidine (HAT) medium. After 3 weeks of culture, the 326 wells with cell growth were examined for antibodies in the culture supernatants as follows. A portion (100 µL) of each culture supernatant was mixed with 10 µL of [³²P]labeled rHuEPO. After incubation of the mixture at 4°C overnight, 10 µL of goat antimouse immunoglobulin (lg) antiserum (Organon Teknika Corp, Westchester, PA) was added. After incubation for two hours more at 4°C, the mixture was centrifuged at 3,000 rpm for 30 minutes to precipitate complexes of [³²P]-labeled EPO and MoAb. The precipitate was rinsed once with PBS and the radioactivity was counted. By such selection, 22 wells were positive. The cells in these wells were transferred into 1-mL cultures in 24-well tissue culture plates and the medium was changed to hypoxanthine-thymidine (HT) medium. After 2 weeks of culture, the culture supernatants were tested again for antibody production. The cells in six positive wells were cloned by the limiting dilution method. Finally, four hybridoma clones (R2, R4, R6, and R12) were selected based on their high reactivity of antibodies to EPO and high production of antibodies in mouse ascitic fluid.

**Preparation and purification of antibodies from mouse ascitic fluid.** MoAbs were produced in the ascitic fluid of BALB/c mice that received injections of hybridoma cells as described previously.6 The MoAbs collected by precipitation with ammonium sulfate were dissolved in and dialysed against 20 mmol/L Tris-HCl, pH 7.9 (buffer A), containing 40 mmol/L NaCl for R2, R4, and R6 and 50 mmol/L NaCl for R12. The dialyzed solution was put on a column containing diethylaminoethyl (DEAE)-cellulose (100 mL/g protein) equilibrated with buffer A containing NaCl at the concentrations listed above. The columns on which the MoAbs R2, R4, and R6 were applied were washed with buffer A containing 100, 40, and 170 mmol/L NaCl, respectively, and then the MoAbs were eluted with buffer A containing 180, 100, or 130 mmol/L NaCl, respectively. MoAb R12 appeared in the flow-through fractions. The purified MoAbs were precipitated by the addition of an equal volume of saturated ammonium sulfate solution and stored at 4°C. All purified MoAbs were homogeneous on SDS-polyacrylamide gel electrophoresis. MoAb concentrations were assayed by measurement of the absorbance at 280 nm and calculation with E₁₄₈₄ assumed to equal 12.

**Antibody subclasses.** The subclass of the purified MoAbs was identified with a Mono ab-ID kit (Zymed Labs, San Francisco) according to the manufacturer’s directions.

**Preparation of immunoadsorbents.** Purified MoAbs were dissolved in and dialysed against 0.2 mol/L NaHCO₃, pH 8, containing 0.3 mol/L NaCl. MoAbs were fixed to Affi-Gel 10 (Bio-Rad, Richmond, CA) according to the procedures reported.14

**Purification of recombinant human erythropoietin.** The culture supernatant of BHK cells producing rHuEPO was obtained as described previously.4 The supernatant (100 L) was filtered with suction and concentrated to 2 L by ultrafiltration on a hollow-fiber device (Amicon DC-10, Danvers, MA) with a nominal Mr cut-off of 10,000. The concentrate was put on an immunosorbent column (4.4 × 6.6 cm) containing 1.5 g of MoAb R2 fixed on Affi-Gel 10 equilibrated with PBS. The column was extensively washed with 5 L of PBS, 800 mL of 10 mmol/L NaPi, pH 7.4, containing 0.5 mol/L NaCl, and 800 mL of 0.15 mol/L NaCl in this order, and eluted by 200 mL of 0.2 mol/L acetate, pH 2.5, containing 0.15 mol/L NaCl. The eluted fraction was neutralized and the protein was concentrated to 20 mL by ultrafiltration with a hollow-fiber device (Mini-Module NM-3, Asahikasei, Tokyo) and was precipitated with 90% ethanol. The precipitate was dissolved in 5 mL of PBS and put on a Sephadex G-100 column (2 × 94 cm) equilibrated with PBS. The column was developed with PBS. The EPO in the fractions (140 to 180 mL) was concentrated and precipitated with 90% ethanol. The precipitate was dissolved in 10 mmol/L NaPi, pH 6.8, containing 0.01 mol/L NaCl and put on a hydroxyapatite column (100 × 7.8 mm; Bio-Rad, Bio-Gel HPH) equilibrated with the same buffer. EPO appeared in the flow-through fractions without being adsorbed.

**Preparation of monoclonal antibodies linked with alkaline phosphatase.** Purified MoAbs (1.4 mg) and alkaline phosphatase from bovine intestinal mucosa (5,000 U. ALP type VII-S, Sigma, St Louis) were mixed in 1.5 mL of PBS and were covalently linked by incubation at room temperature for one hour in the presence of 0.2% glutaraldehyde. The mixture was dialyzed against 50 mmol/L Tris-HCl buffer, pH 8.0, containing 1 mmol/L MgCl₂ overnight at 4°C with two changes of the buffer. To the dialyzed solution, BSA and NaN₃ were added at the final concentrations of 1% and 0.02%, respectively, and stored in the dark at 4°C.

**Enzyme-linked immunosorbent assay.** By the use of MoAbs R2 and R6, which bind to EPO at different sites, we developed an ELISA for EPO. EPO was sandwiched between R6 fixed in microtiter wells and the enzyme-linked R2. All procedures were done at
room temperature (−25°C). The MoAb R6 solution (10 μg/mL in 50 mmol/L sodium carbonate buffer, pH 9.6, containing 0.02% NaN3) was added to the wells (100 μL per well) of microtiter plates with 96 flat-bottomed wells (Falcon no. 3912, Oxnard, CA) and incubated for two hours. The unfixed MoAb was removed by washing three times with PBS containing 0.05% Tween 20 and 0.02% NaN3 (washing buffer). To each well, 200 μL of PBS containing 1% BSA was added and the mixture incubated for two hours to block extra binding sites for protein. After washing three times with washing buffer, 100 μL/well of a standard EPO solution (0 to 120 mU/mL in PBS containing 5% BSA, 0.05% Tween 20, 1 mmol/L EDTA, and 0.02% NaN3) or the sample solution (mixtures of 90 μL of plasma and 10 μL of PBS containing 0.5% Tween 20, 10 mmol/L EDTA, and 0.2% NaN3) was added to the wells, which were incubated for two hours to form the fixed R6 - EPO complexes. To the wells washed three times with washing buffer, 100 μL/well of the alkaline phosphatase-linked MoAb R2 solution, which was diluted 200-fold with washing buffer, was added and the wells were incubated for two hours to form ternary complexes (fixed R6 - EPO - enzyme-linked R2). The wells were washed five times with washing buffer and then 100 μL of 2 mg/mL p-nitrophenyl phosphate disodium (Sigma, St Louis) was added. After incubation for 30 minutes to allow the enzyme reaction to proceed, the reaction was stopped by the addition of 50 μL of 3 mol/L NaOH. The optical density at 405 nm was measured with a Microtiter Reader (Nippon Inter Med, Immuno Reader NJ-2000, Tokyo). This procedure is referred to as a two-step reaction, because EPO was first bound to the fixed MoAb R6 and then the enzyme-linked MoAb R2 was added to form the ternary complexes. In the procedure referred to as a one-step reaction, 100 μL of the mixture of the EPO solution and the enzyme-linked MoAb R2 solution was added to the well in which MoAb R6 had been fixed, for a quicker procedure.

In the ELISA experiments in which the question of whether MoAbs recognize different epitopes on EPO was considered, each MoAb, R2, R4, R6, and R12, was fixed in wells and then incubated with rHuEPO (0 to 1 μg/mL) to form complexes of fixed MoAb and EPO. Each alkaline phosphatase-linked MoAb was added to each well so that every combination between the fixed MoAbs and the enzyme-linked MoAbs could be made. The formation of complexes of R6, EPO, and enzyme-linked R2 was detected by measurement of enzyme activity. All conditions including washing and incubation were as described above.

RESULTS

Binding of monoclonal antibodies to erythropoietin. By use of rHuEPO produced by BHK cells as an antigen, four hybridoma clones (R2, R4, R6, and R12) that were stable in their rapid growth and their production of MoAbs to EPO were isolated. The subclass of all antibodies identified with antitumor antibodies that were enzyme linked and specific for a subclass was IgG1, γκ. Binding of the MoAbs to rHuEPO was studied; increasing concentrations of a given antibody were incubated with a fixed amount of 125I-labeled EPO and the 125I-labeled EPO - MoAb complex was precipitated by the use of antitumor Ig antiserum (Fig 1). The maximum amount of 125I-labeled EPO that could be precipitated by the MoAbs R2, R4, or R6 was approximately 75% of the total ligand. The apparent dissociation constant, kd, was calculated from the MoAb concentration required to bind 50% of the maximum amount of bindable 125I-labeled EPO (Table 1). Three MoAbs, R2, R4, and R6, bound to EPO with high affinities, with kd = 2, 4, and 1 nmol/L, respectively; the affinity of R12 was about 1/100 that of the others.

The kd values were estimated by another method, competition binding assay; the incubation mixtures of the formation of complexes of 125I-labeled EPO and MoAb contained 125I-labeled EPO and a given MoAb at fixed concentrations, and different amounts of unlabeled EPO. The 125I-labeled EPO - MoAb complexes were precipitated and the precipitated radioactivity was counted. Scatchard plots gave straight lines (Fig 2), indicating that the affinity of EPO to the MoAb was unchanged before and after iodination. The kd values (Table 1) obtained from these plots were in good agreement with those obtained by the radioimmunoassay technique, indicating that the Scatchard plot of the MoAb was linear in this range.

<p>| Table 1. Dissociation Constants of Binding of Monoclonal Antibodies to Recombinant Human Erythropoietin |
|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>MoAb Concentration</th>
<th>Radioimmuno-precipitation*</th>
<th>Competitive Radioimmuno-precipitation†</th>
</tr>
</thead>
<tbody>
<tr>
<td>R2</td>
<td>1.7</td>
<td>2.0</td>
</tr>
<tr>
<td>R4</td>
<td>4.0</td>
<td>3.2</td>
</tr>
<tr>
<td>R6</td>
<td>1.1</td>
<td>0.8</td>
</tr>
<tr>
<td>R12</td>
<td>240</td>
<td>—</td>
</tr>
</tbody>
</table>

*From Fig 1.†From Fig 2.
Inhibition of binding of MoAbs to $^{125}$I-labeled rHuEPO by unlabeled EPO (Scatchard plots). The procedures to precipitate the MoAb-EPO complexes were the same as those described in the legend of Fig 1 except that the mixtures for formation of the immunocomplexes contained 0.14 ng (22,700 cpm) of $^{125}$I-labeled rHuEPO, 0.1 μg of MoAb, and 0 to 100 ng of unlabeled rHuEPO. O, R2; △, R4; △, R6. Each point is the mean of triplicate samples. r is $[^{125}$I-labeled EPO/MoAb]/[free $^{125}$I-labeled EPO] and c is $\alpha/(1 + r)$ where $\alpha$ is the total EPO (ng/mL). The kd values were calculated from the slopes of the straight lines.

**Fig. 2.**

**Binding of monoclonal antibodies to the fully deglycosylated erythropoietin.** rHuEPO produced by BHK cells has N-linked and O-linked oligosaccharides (Tsuda et al, unpublished observations). To know if EPO-bound carbohydrates were involved in the interaction of MoAb and EPO, rHuEPO was fully deglycosylated with N-glycanase, neuraminidase, and endo-α-N-acetylgalactosaminidase. Completion of the enzyme reactions was confirmed with SDS-polyacrylamide gel electrophoresis; the native EPO migrated with 37 Kd and the deglycosylation resulted in appearance of a band at 18 Kd, which corresponded to the molecular mass of the protein backbone deduced from the DNA sequence.17 The reactivity of the deglycosylated EPO to MoAbs was tested by its binding to each MoAb fixed in the microtiter wells; the EPO was labeled with $^{125}$I and incubated with different amounts of the fixed MoAbs (Fig 3). Experiments with glycosylated $^{125}$I-labeled rHuEPO were done for comparison. The specific binding of both $^{125}$I-labeled EPOs increased as the amounts of the fixed MoAbs increased, indicating that carbohydrates attached to EPO were not part of the epitopes recognized by the MoAbs. Comparison of binding curves with glycosylated and deglycosylated EPO showed that deglycosylation of EPO did not change its affinities to MoAbs R2 and R4 but increased affinities to MoAbs R6 and R12. The binding affinity of the MoAbs to fully deglycosylated and native EPOs. All procedures were done at room temperature. The solutions containing MoAbs at the concentrations specified on the abscissa were added to flat-bottom separable microtiter wells (100 μL/well) and incubated for two hours. The excess MoAb was removed by washing with the washing buffer (see text). To each well, 200 μL of PBS containing 1% BSA was added and incubated for two hours to block extra binding sites for protein. After washing three times with washing buffer, 50 μL of PBS containing 0.1% BSA and the native $^{125}$I-labeled rHuEPO (82,000 cpm, 127 μCi/μg) or the fully deglycosylated $^{125}$I-labeled rHuEPO (47,000 cpm, 77 μCi/μg) was added to the well and incubated for two hours. The unbound ligand was washed away and the radioactivity of the wells was counted. O, R2; △, R4; △, R6; ◇, R12. (——), native $^{125}$I-labeled rHuEPO; (-----), deglycosylated $^{125}$I-labeled rHuEPO. T represents the total radioactivity added; B, bound radioactivity; and $B_o$, bound radioactivity found in the absence of MoAb (control value).
MONOCLONAL ANTIBODIES TO ERYTHROPOIETIN

固定的MoAb R2对EPO的抑制作用比固定MoAb R6的抑制作用更高，尽管反向的结合为自由MoAbs（表1）；见讨论部分。

抗原决定簇。试图找出EPO定向的MoAbs识别不同的表位。实验中使用ELISA系统描述的材料和方法部分。每个MoAb固定在酶联微孔板上。固定EPO的洗去，并且一个MoAb固定，与上述注册物固定绑定。凝集酶反应中，MoAbs被R2 (O), R4 (△), R6 (△), 和 R12 (●). Figure 4 显示结果。当MoAb R2, R4, 或 R12被固定在微孔板（图4A, B, and D, 分别），酶活性仅与凝集酶被固定MoAb R6。然而，酶活性的出现包括MoAb R6和酶联凝集酶MoAb R2, R4, and R12 (Fig 4C)。活性未被检测，与预期，与每一个组合的凝集酶的MoAb和它的酶联凝集酶的对应物。这些结果表明，MoAb R6对EPO的抑制作用发生在不同的区域，但与其它MoAbs的区域不同。

Table 2. Neutralization of Erythropoietin Activity by Monoclonal Antibodies

<table>
<thead>
<tr>
<th>Erythropoietin Activity (%)</th>
<th>R2</th>
<th>R4</th>
<th>R6</th>
<th>R12</th>
</tr>
</thead>
<tbody>
<tr>
<td>rHuEPO</td>
<td>60</td>
<td>80</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>Human urinary EPO</td>
<td>45</td>
<td>88</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Human serum</td>
<td>32</td>
<td>67</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat serum</td>
<td>10</td>
<td>31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse serum</td>
<td>32</td>
<td>67</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabbit serum</td>
<td>18</td>
<td>99</td>
<td></td>
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</tr>
</tbody>
</table>

In vitro EPO activity was assayed by the use of stimulation of [3H]-thymidine into DNA in cultured liver cells of fetal mice. EPO activity found in the absence of MoAb was defined as 100%. EPO concentrations in the assay mixtures were 100 mU/mL for rHuEPO, 50 mU/mL for human urinary EPO, 40 mU/mL for human serum, 20 mU/mL for rat serum, 15 mU/mL for mouse serum, and 55 mU/mL for rat serum. MoAbs were added to the assay mixtures without incubation with EPOs.
Table 3. Inhibition of 125I-Labeled Erythropoietin Binding to EPO by Monoclonal Antibodies

<table>
<thead>
<tr>
<th>Addition</th>
<th>Amount (μg)</th>
<th>Bound 125I-labeled EPO (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>—</td>
<td>4,135</td>
</tr>
<tr>
<td>Unlabeled rHuEPO</td>
<td>2.5</td>
<td>451</td>
</tr>
<tr>
<td>Control MoAb†</td>
<td>5</td>
<td>4,276</td>
</tr>
<tr>
<td>R2</td>
<td>5</td>
<td>915</td>
</tr>
<tr>
<td>R4</td>
<td>20</td>
<td>350</td>
</tr>
<tr>
<td>R6</td>
<td>5</td>
<td>1,381</td>
</tr>
<tr>
<td>R12</td>
<td>5</td>
<td>4,045</td>
</tr>
</tbody>
</table>

*Additions were made to the binding assay mixtures containing target cells just before the addition of 125I-labeled rHuEPO.
†The control MoAb was MoAb (lgG) against transfused EPO.

MoAbs was tested with an erythroleukemia cell line, TSA8, and also mouse fetal liver cells that both had receptors specific for EPO. The results with TSA8 cells are shown in Table 3. Similar results were obtained by the use of fetal liver cells. The presence of 2.5 μg (400-fold) unlabeled EPO decreased the bound radioactivity to 13% of the total; thus, 87% of the total bound radioactivity was due to the specific binding of the ligand to its receptors. MoAbs against EPO inhibited the binding of 125I-labeled EPO to its receptor, and the control MoAb against transfused EPO had no effect on the binding of the ligand. Thus, it seemed that MoAb - EPO complexes had no or greatly decreased affinity to EPO receptor; this was clearly seen with the R2 - EPO and R6 - EPO complexes.

**Purification of recombinant human erythropoietin with monoclonal antibodies.** Human urinary EPO-directed MoAb from a hybridoma established in this laboratory was effective in the purification of EPO from human urine and culture supernatants of BHK cells that produced rHuEPO, but pretreatment of the EPO with SDS was needed for EPO to bind with the immobilized MoAb. To develop a more rapid purification procedure, the new MoAbs obtained here were examined for suitability for this purpose. In pilot experiments, concentrates from the culture supernatant of BHK cells were put on columns that contained similar amounts of each MoAb fixed on Affi-Gel 10, and then the EPO activity in the flow-through fractions and the fractions eluted at pH 2.5 was measured. Most of the EPO applied was adsorbed by the immobilized MoAbs R2, R4, R6, and R12. When the eluted fractions were tested for EPO activity, the highest recovery (66%) was obtained with MoAb R2. The activity recovered in eluted fractions with MoAbs R4, R6, and R12 was 34%, 19%, and 40%, respectively.

The large-scale purification of rHuEPO in the supernatant of BHK cells was attempted by the use of a column containing fixed MoAb R2 (see the Materials and Methods section). Purification with the immunosorbent column was effective (Table 4); most of the protein and little EPO activity in the concentrate of culture supernatants emerged in the flow-through fractions without being adsorbed, and EPO was eluted sharply at pH 2.5. About 2,800-fold purification was achieved by this single step, and 84% of the activity was recovered. Subsequent chromatography with Sephadex G-100 and then hydroxyapatite yielded purified EPO with 3,260-fold purification and 52% recovery of the starting activity. The purified EPO had the specific activity of 137,000 U/A₂₈₀nm, which was higher than that of the EPO purified with an earlier preparation of an MoAb. The final EPO preparation was pure on SDS-polyacrylamide gel electrophoresis and high-pressure liquid chromatography. The immunosorbent containing the immobilized R2 could be used 30 times without any deterioration.

**Enzyme-linked immunosorbent assay.** The above finding that the antigen site recognized by MoAb R6 differed from the sites recognized by other MoAbs prompted us to develop a sandwich-type ELISA for the measurement of EPO levels. MoAb R6, with the highest affinity, was fixed in the microtiter wells. In the two-step reaction, EPO in samples was bound to the fixed R6 and then alkaline phosphatase-linked R2 was added to the wells to form ternary complexes of R6, EPO, and the enzyme-linked R2. In the alternative procedure, the one-step reaction, mixtures of EPO samples and enzyme-linked MoAb R2 were added to the wells coated with MoAb R6. After the unbound enzyme-linked R2 was removed, the enzyme activity of the wells was measured. The relationship of the amounts of rHuEPO and the enzyme activity was linear regardless of different procedures (Fig 5); use of the one-step reaction shortened the time needed for completion of the process to seven hours. Once the microtiter wells in which the first antibody, R6, and BSA have been fixed, results can be obtained within three hours.

To check the reproducibility of the ELISA, an intraassay study in which four samples containing EPO at different concentrations were assayed by six runs at one time was done (Table 5). An interassay study also was done; here, triplicate samples of each rHuEPO solution were assayed on six study days (Table 6). In both studies, the coefficients of variation were satisfactory (<20%).

We have developed RIA for EPO by the use of antisera against human urinary EPO and measured EPO levels in plasma samples from normal subjects and anemic or polycythemic patients. These plasma samples were stored at —80°C. The stored samples from four normal male subjects were assayed by the use of antisera against human urinary EPO and measured EPO levels in plasma samples from normal subjects and anemic or polycythemic patients. These plasma samples were stored at —80°C. The stored samples from four normal male subjects...
**MONOClonal Antibodies to Erythropoietin**

![Graph](image)

**Fig 5.** Enzyme-linked immunosorbent assay of EPO. ELISA was done as described in the Materials and Methods section by use of the purified rHuEPO. 0, one-step reaction in which mixtures of EPO and alkaline phosphatase-linked R2 were added to wells coated with R6; O, two-step reaction in which EPO and the enzyme-linked R2 were added stepwise to the wells. Each point is the mean of triplicate samples.

were serially diluted and EPO levels in the diluted samples were assayed with ELISA. As shown in Fig 6, there was a linear relationship between EPO levels and dilution of the samples. Such relationship existed for all samples from eight normal subjects. EPO levels in these 12 samples ranged between 29 mU/mL and 7 mU/mL with a mean of 15.7 ± 7 (SD).

Of the stored plasma samples from normal subjects and patients, 38 samples were selected and their EPO levels were measured with ELISA to compare with those found by the use of R1A. The results are shown in Fig 7 and indicate a high degree of correlation between these two methods, with a correlation coefficient of 0.965.

![Graph](image)

**Fig 6.** Correlation between EPO levels in human plasma samples and dilution of the samples. Four plasma samples from normal subjects were serially diluted by PBS containing 5% BSA and EPO levels in the diluted samples were assayed with ELISA by the use of a two-step reaction (see the Materials and Methods section). The enzyme activity found for each sample was converted to the quantity of EPO by the use of a standard curve shown in Fig 5. Each point is the mean of triplicate samples.

**Table 5.** Intraassay Variance of Enzyme-Linked Immunosorbent Assay

<table>
<thead>
<tr>
<th>Sample</th>
<th>No. of Runs</th>
<th>Mean EPO (mU/mL)</th>
<th>SD</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6</td>
<td>12.6</td>
<td>1.3</td>
<td>10.7</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>38.4</td>
<td>2.2</td>
<td>5.7</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>71.6</td>
<td>5.6</td>
<td>7.7</td>
</tr>
<tr>
<td>4</td>
<td>6</td>
<td>90.7</td>
<td>5.9</td>
<td>6.5</td>
</tr>
</tbody>
</table>

Abbreviation: CV, coefficient of variation.

Four sample solutions containing rHuEPO at different concentrations were assayed with ELISA by a one-step reaction (see the Materials and Methods section). The mean EPO is the mean of sextuplicate assays of each sample.

**DISCUSSION**

Here we described properties of the interaction of EPO-directed MoAbs with their antigen, and the use of MoAbs with properties suitable for purification of EPO and for assay of EPO with sandwich-type ELISA. Of the four MoAbs obtained by the use of rHuEPO as antigen, three (R2, R4, and R6) bound to the antigen with high affinities in the nanomolar range. The results of EPO binding to the MoAbs...
that required SDS-treatment of EPO for antibody-antigen interaction. The SDS-treatment might damage structure of the EPO.

Experiments done to distinguish between the epitopes recognized by the MoAbs (Fig 3) showed that the antigen site(s) bound to MoAb R6 differed from the sites bound by other MoAbs. There is still a possibility that the other MoAbs (R2, R4, and R12) bound at different site(s) on the antigen; location of the epitope(s) of a given MoAb in the vicinity of that recognized by another MoAb would inhibit formation of the ternary complex MoAb - EPO - MoAb. It is also possible that binding of an MoAb to the antigen induces a conformational change of the antigen, burying the epitope(s) so that it becomes inaccessible to other MoAbs. The findings that the MoAbs R6 and R2 bound to EPO with high affinities and recognized different antigenic determinants made it possible to develop a sensitive and easier-to-handle method for EPO assay. So far, the RIAs to assay EPO have been developed by the use of antisera. The sandwich-type ELISA developed here by the use of MoAbs had advantages over the RIAs in practicality, although the ELISA was comparable with the RIAs in sensitivity; one can assay EPO for a short time without using any radioactive compounds and the assay conditions, once established, can be used semipermanently, because MoAbs with the constant properties can be supplied continually. Our sandwich-type ELISA should be useful to assay EPO levels in human plasma for investigating an etiology of defective erythropoiesis and also to monitor rHuEPO given to anemic patients.

The MoAbs inhibited the biological activity of EPO from human urine and these activities in plasma of humans, rats, mice, and rabbits as well as the activity of the antigen, rHuEPO (Table 2). This inhibition seemed to be due to blockage of EPO binding to the target cells by MoAbs, because 125I-labeled rHuEPO binding to the cells was mostly eliminated by MoAbs (Table 3). This observation did not lead us to conclude that the MoAbs bind with EPO at its receptor binding domain(s), because blockage of EPO binding by MoAbs may result from conformational changes of EPO induced by interaction with the MoAbs. This argument is valid for the antibodies reported by Sytkowski and Donahue, who raised rabbit polyclonal antibodies with synthetic peptide fragments in the EPO used as the antigen. Considerations must wait until the EPO sequences recognized by antibodies are identified and the corresponding peptides are found to compete with EPO in binding to the EPO receptor. This approach is currently under investigation in this laboratory.

REFERENCES


Characterization and use of monoclonal antibodies directed against human erythropoietin that recognize different antigenic determinants

M Goto, A Murakami, K Akai, G Kawanishi, M Ueda, H Chiba and R Sasaki