Isolation of Human Erythropoietin with Monoclonal Antibodies*

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Human erythropoietin was isolated from urine of aplastic anemic patients in a high yield with a simple purification procedure using an immunoadsorbent column of monoclonal antibodies and a Sephadex G-100 column. About 6 mg of erythropoietin was isolated from 700 liters of urine and the specific activity was estimated to be 81,600 units/mg of protein with an in vivo 59Fe incorporation assay method, using starved rats. Activity measurement of the extracts from sliced gels after sodium dodecyl sulfate-polyacrylamide gel electrophoresis and the Western blotting technique revealed heterogeneity of the isolated erythropoietin, which is probably caused by variable amounts of carbohydrates attached to the polypeptide chain. Thirty amino acids in the NH2-terminal portion of the isolated hormone were sequenced.

Erythropoietin is a sialoglycoprotein which is believed to play an important role in regulating, by stimulating, erythropoiesis. Purification of erythropoietin from the urine of patients with aplastic anemia, using conventional purification procedures, has been reported (1, 2). However, laborious purification procedures with low yields and limited supplies of starting material have prevented us from obtaining amounts of pure erythropoietin suitable for studying its structure, mechanism of action, and metabolism. We have prepared a stable hybridoma clone that secretes monoclonal antibody against human urinary erythropoietin.1 We describe here a very simple isolation procedure with a high yield of erythropoietin from human urine by the use of this monoclonal antibody.

EXPERIMENTAL PROCEDURES

Materials—Erythropoietin (38,000 units/mg of protein with in vivo assay method) was purified from human urine by a combination of conventional methods, immunoadsorbents column against contaminants and preparative SDS-polyacrylamide gel electrophoresis as a final step. The hybridoma secreting monoclonal antibody against erythropoietin was prepared by fusion of mouse myeloma cells with spleen cells from a mouse receiving the first immunization with the erythropoietin antigen in SDS-polyacrylamide gel suspension; the gel after SDS-polyacrylamide gel electrophoresis for the final step of erythropoietin purification was sliced and the sliced gel containing erythropoietin protein was ground. The animal was boosted with the purified erythropoietin. A large amount of the monoclonal antibody (IgG) against erythropoietin was purified from mouse ascitic fluids. Further details of all mentioned above have been described elsewhere.2 The antibody was fixed on Affi-Gel 10 (3). Urine, collected from anemic patients, was filtered under suction, and concentrated by ultrafiltration on a hollow-fiber device (Amicon) with a nominal M cutoff of 10,000. The urine concentrate was lyophilized. Standard erythropoietin (sheep plasma erythropoietin step III, 4 units/mg of protein) was obtained from Connaught Medical Research Laboratories, Willowdale, Canada.

Assay for Erythropoietin—For selection of urine from anemic patients with high erythropoietin titers, the activity was assayed by counting erythroblast colonies (4). Erythropoietin was assayed in vitro, utilizing the stimulatory effect of erythropoietin on incorporation of 59Fe into heme (5) or [3H]thymidine into DNA (6) in cultured mouse fetal liver cells. Each value shown in this paper is the mean of triplicate cultures. The erythropoietin activity was determined also in vivo using the starved rat (4 rats/sample) (7).

SDS-Polyacrylamide Gel Electrophoresis and Erythropoietin Activity in Gel Extracts—SDS-polyacrylamide gel electrophoresis was performed by the method of Laemmli (8) and stained with silver according to the manufacturer's directions (Bio-Rad). In some cases, erythropoietin activity of extracts from sliced gels was measured. The gel without staining was sliced into 1-mm lengths. The gel pieces were put into test tubes containing PBS, pH 7.4, 0.1% bovine serum albumin (0.5 ml/sliced gel) and ground with a glass rod. After incubating the suspensions overnight to extract protein, they were centrifuged to obtain the clear supernatants. The activity in the supernatants was measured with an in vitro [3H]thymidine method (6).

Amino Acid Sequence—Amino acid sequence of pure erythropoietin was determined with an Applied Biosystems 470A automatic sequenator, a gas phase sequenator (9).

RESULTS

Purification of Erythropoietin from Urine Concentrate—A concentrate from about 700 liters of urine was applied on a column in which erythropoietin-directed monoclonal antibody fixed on Affi-Gel 10 was packed. The column was extensively washed and then developed with the pH 2.5 buffer. As shown in Fig. 1, purification with the immunoadsorbent column was tremendously effective; most of the protein in the urine concentrate emerged in the flow-through fractions without being adsorbed, and erythropoietin was eluted sharply by the pH 2.5 buffer. About 2600-fold purification was achieved by this single step and 75% of the activity was recovered (Table I). The presence of SDS in heat treatment of the urine concentrate is needed for erythropoietin to be retained on the column. Erythropoietin mostly appeared in the flow-through fractions on the heat treatment in the absence of SDS. Activity measurement after dilution of the SDS-treated urine concentrate with PBS or the erythropoietin assay medium showed little loss of activity during the SDS treatment; since the activity was measured with the SDS-treated urine concentrate diluted more than 1000-fold, effects of SDS on the target cells (fetal mouse liver cells) were avoided. Erythropoietin in the concentrate diluted back to the original urine volume nor that in the urine before concentration was adsorbed on the column unless the sample was subjected to heat treatment in the presence of SDS. Erythropoietin eluted from
FIG. 1. Purification of erythropoietin from human urine concentrate with an immunoadsorbent column containing monoclonal antibody against erythropoietin. ○, absorbance at 280 nm; □, erythropoietin activity assayed with in vitro [3H]thymidine incorporation method. The lyophilized urine concentrate (350 g) was dissolved in 700 ml of PBS, pH 7.4, and dialyzed extensively. Insoluble materials were removed by centrifugation and the supernatant (680 ml) was heated at 100 °C for 3 min after the addition of solid SDS (final concentration 2%). The SDS-treated solution was cooled and left overnight at 0 °C. The precipitated SDS was removed by centrifugation and the supernatant (580 ml) was loaded on an immunoadsorbent column (3.2 x 2.5 cm) equilibrated with the PBS containing 716 mg of the erythropoietin-directed monoclonal antibody fixed on Affi-Gel 10. The column was extensively washed with 1 liter of the PBS, 500 ml of 10 mM NaCl, pH 7.4, 0.5 M NaCl, and 500 ml of 0.15 M NaCl in this order, and then eluted by reverse flow of 0.2 M acetate, pH 2.5, 0.15 M NaCl. The eluted fractions (112 ml) were immediately neutralized by adding 5.3 ml of 3.4 M Tris. The flow rate was 24 ml/h during application of the sample and elution with the pH 2.5 buffer, while it was 200 ml/h during washing of the column. The volume of one fraction was 8 ml.

TABLE I

<table>
<thead>
<tr>
<th>Protein</th>
<th>Activity in vitro</th>
<th>Yield</th>
<th>Specific activity in vitro</th>
<th>Purification</th>
<th>Specific activity in vivo</th>
</tr>
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<tbody>
<tr>
<td>Urine concentrate</td>
<td>34 x 10^3</td>
<td>792</td>
<td>100</td>
<td>3</td>
<td>14</td>
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<tr>
<td>Immunoadsorbent column</td>
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<tr>
<td>Sephadex G-100</td>
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</tr>
<tr>
<td></td>
<td>1</td>
<td>594</td>
<td>75</td>
<td>59,400</td>
<td>2,580</td>
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<tr>
<td></td>
<td>5.7</td>
<td>500</td>
<td>63</td>
<td>88,000</td>
<td>3,830</td>
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the immunoadsorbent column was no longer adsorbed on a newly made column but when subjected to the SDS treatment it was adsorbed again.

Erythropoietin purified with the immunoadsorbent column was recognized as a main band with M, 35,000 on SDS-polyacrylamide gel electrophoresis, but some other protein bands, including a M, 20,000 protein which was a main contaminant, were seen (Fig. 2, lane 4). Furthermore, this preparation was faintly tinged with brown.

Further purification with a Sephadex G-100 column revealed two peaks of protein (Fig. 3A). It was found by measuring the activity in the pooled preparation of each peak that most of the erythropoietin activity was recovered in the second peak (Table I) and that about 5% of the total recovered activity appeared in the void fractions (the first peak). The faint brown appeared in the void fractions, while the pooled erythropoietin preparation was quite transparent. Fig. 3B shows the analytical results of the Sephadex G-100 fractions on SDS-polyacrylamide gel electrophoresis. Contaminants including the main contaminant, the M, 20,000 protein, which were found in the erythropoietin preparation purified with the immunoadsorbent column, were not detected in any fractions of the second peak (Fig. 3B, lanes 43–53). As is most clearly seen in fraction 48 (Fig. 3B, lane 48), however, they contained faint but detectable protein components on the leading side of the erythropoietin main band with M, 35,000. Activity measurements in the extracts from sliced gels indicated that there is erythropoietin activity in the leading side as well as in the main band (Fig. 3C), suggesting heterogeneity of the erythropoietin protein. It was proved with the Western blotting technique that the components in the leading side are indeed erythropoietin protein; the monoclonal antibody against erythropoietin reacted with these components (Fig. 4, lane 3). The erythropoietin protein with M, 35,000 binds properly with the antibody. Protein species which migrated somewhat faster than the erythropoietin main band and bound with the antibody were also present in the erythropoietin preparation before Sephadex G-100 column chromatography (Fig. 4, lane 2). Thus, the final erythropoietin preparation contains erythropoietin species with mobilities larger than the M, 35,000 erythropoietin, although at only 5–10% of the amount of M, 35,000 erythropoietin protein, based on
the clear solution was loaded on a Sephadex G-100 column (1.2 erythropoietin fraction eluted from the immunoadsorbent column which we purified and used as
d. The dried material was dissolved in
fraction purified with the immunoadsorbent column.

cm) equilibrated with the PBS and the column was developed at thymidine incorporation method.

the fractions of Sephadex G-100 chromatography.

with a speed of 6 ml/h. The volume of one fraction was

so that the main component in the void fractions was M, 35,000 protein. Furthermore, it was shown that this component was indeed erythropoietin protein; erythropoietin activity was found in the extracts from sliced gels containing the M, 35,000 component after SDS-polyacrylamide gel electrophoresis of fraction 36 (not illustrated) and Western blotting revealed the binding of this component with the monoclonal antibody against erythropoietin (Fig. 4, lane 4). Interestingly, the M, 20,000 protein did not bind with the antibody. Activity measurement of fraction 36, in which there should be no contamination of erythropoietin from the second peak, showed about 1/3% of the specific activity of the second peak, based on absorbance at 280 nm. More than 50% of the protein of fraction 36, however, appears to be the M, 35,000 protein (Fig. 3B, lane 36) from the intensity of protein staining. Therefore, it seems that erythropoietin molecules in the void fractions have much lower activity than those in the second peak (see “Discussion”). In spite of the occurrence of the undefined erythropoietin species on Sephadex G-100 chromatography, purification of human urinary erythropoietin with an immunoabsorbent and a Sephadex G-100 column provides pure erythropoietin in a high yield (Table I).

Characterization of the Purified Erythropoietin—Protein concentrations were determined with a Coomassie brilliant blue binding assay according to the method of Bradford (10). A value of $E_{280\text{nm}}$ for pure erythropoietin was calculated to be 1.31 when a standard curve was made with ovalbumin and 19.7 with bovine serum albumin. The former value was adopted here because of the unusual behavior of bovine serum albumin in this assay method. A value of $E_{1\%_{280\text{nm}}}=12.6$ was also obtained by the method of Lowry et al. (11) using bovine

silver-staining intensity. The erythropoietin preparation, which we purified and used as an immunogen for raising erythropoietin-directed hybridomas, also contained such erythropoietin species (Fig. 4, lane 1). Treatment with sialidase converted the main M, 35,000 band to one with larger mobility (Fig. 4, lane 5), which suggests that electrophoretic heterogeneity of erythropoietin is, at least in part, due to its variable amount of sialic acid attached to erythropoietin protein.

The main M, 20,000 contaminant and others in the erythropoietin preparation eluted from the immunoabsorbent column appeared in the void fractions on Sephadex G-100 chromatography (Fig. 3B, lanes 36–38). It was rather surprising...
serum albumin as standard. The specific activity of purified erythropoietin was determined to be 88,000 units/mg of protein with an in vitro [3H]thymidine incorporation method and 81,800 with an in vivo starved rat method (see Table I). Assay with the in vitro 35S incorporation method gave a similar value. A specific activity of 70,400 units/mg of protein has been reported for erythropoietin isolated from human urine with conventional purification procedures by Miyake et al. (2) using $E_{280}^{1\%} = 8.5$.

Sialidase treatment (see legend of Fig. 4) of our erythropoietin completely abolished in vivo activity, while in vitro activity increased 1.3-fold. Similar results have been reported with erythropoietin partially purified from the plasma of anemic sheep (12, 13). Loss of in vivo activity would be caused by the hepatic removal of asialoglycoproteins from the circulation (14).

Thirty amino acids in the NH$_2$-terminal portion of erythropoietin were sequenced with a gas phase sequenator; the sequence was H$_2$N-Ala-Pro-Arg-Leu-Ile-Leu-Asp-Ser-Arg-Val-Leu-Glu-Arg-Tyr-Leu-Leu-Glu-Ala-Lys-Glu-Ala-Glu-?-Ile-Thr-Asp-Gly-Gly-Ala. Possible candidates for the unidentified positions are cysteins involved in disulfide bonds or amino acids (probably Asn, from the carbohydrates contained$^3$) to which carbohydrate residues were attached. No significant amount of amino acids other than alanine was detected as the NH$_2$-terminal residue, and therefore, heterogeneity of the purified erythropoietin found on SDS-polyacrylamide gel electrophoresis (see Fig. 4) is presumably due to difference in carbohydrate residues attached to the erythropoietin polypeptide chain. The putative 26 NH$_2$-terminal amino acids of erythropoietin were recently described by Sue and Sytokowski (15), i.e., H$_2$N-Ala-Pro-Arg-Leu-Ile-Asn-Asp-Ser-Arg-Val-Leu-Glu-Arg-Tyr-Leu-Leu-Glu-Ala-Lys-Glu-Ala-Glu-Lys-Ile-Thr. Three amino acids, positions 3, 7, and 24, differ from our sequence for unknown reasons.

**DISCUSSION**

A hybridoma secreting the monoclonal antibody against human urinary erythropoietin has been raised (17) but the paper presented here is the first report of the use of monoclonal antibody for the large-scale isolation of erythropoietin. Rapid isolation of erythropoietin from human urinary concentrate with a high yield can be achieved by using an immunoadsorbent column packed with the monoclonal antibody and a Sephadex G-100 column. Adequate amounts of pure erythropoietin will make possible further studies on the biochemistry and physiology of erythropoietin and its clinical testing.

The immunoadsorbent column does not bind erythropoietin in the untreated urine concentrate but binds erythropoietin in the SDS-treated one. Treatment with SDS was needed for the purified erythropoietin to be retained again on the immunoadsorbent column. It appears, therefore, that the lack of binding of erythropoietin in the untreated urine concentrate with the antibody is not attributable to the interaction of the erythropoietin with other components in the concentrate but to a property of the antibody. The hybridoma secreting this antibody was constructed by using spleen cells from a mouse receiving its primary immunization with an antigen containing ground SDS-polyacrylamide gel. Therefore, this antibody may be directed against an epitope which is buried in the interior of the native erythropoietin molecule, inaccessible to the antibody, but exposed in the presence of SDS. It is also possible that the antibody recognizes a structure formed by the binding of SDS to erythropoietin. The binding of erythropoietin to the immunoadsorbent column can occur only in the presence of SDS but there appears to be no problems concerning the eluted erythropoietin. The purified erythropoietin no longer binds with the antibody, and has a specific activity comparable to the value for pure erythropoietin (2). The immunoadsorbent column is so extensively washed before the elution of the erythropoietin that, if SDS remains in the eluted erythropoietin, the amount would be very small.

The presence of the erythropoietin molecule with a low specific activity in the void fractions of Sephadex G-100 chromatography is puzzling. The void fractions may contain associated forms of erythropoietin and those of the contaminant, M, 20,000 protein which has a high affinity for mouse IgG (for instance, a component of compliments). Association of erythropoietin may result in reducing the activity. It seems, however, that the binding of the M, 20,000 protein to the antibody column is mediated by erythropoietin, since the M, 20,000 protein fractionated on the gel electrophoresis was unable to bind with the monoclonal antibody (see Fig. 4, lane 4). An intriguing hypothesis is that the occurrence of high molecular weight species of erythropoietin with reduced activity is caused by association of erythropoietin with the M, 20,000 protein which is a physiological regulator of erythropoiesis. Existence of the inactive erythropoietin species with a high molecular weight, which dissociates to produce the active hormone, has been suggested (18). Analysis of the erythropoietin in the void fractions is in progress. With conventional purification procedures, such species of erythropoietin must in all likelihood have escaped detection due to its low activity.

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