High-Level Expression and Purification of a Recombinant Human Erythropoietin Produced Using a Baculovirus Vector

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Conditions presently have been established for the high-level expression and simplified purification of recombinant human erythropoietin produced in Spodoptera frugiperda cells. Expression, as mediated by infection with a recombinant baculovirus, was accomplished in suspension culture using reduced levels of serum and media supplements experimentally determined to provide optimum levels of factor production (500,000 U/L). Purification of this recombinant human erythropoietin to virtual homogeneity (≥99%) was accomplished via a simple three-step procedure involving isocratic elution from DEAE-Sephacel, reverse-phase high performance liquid chromatography (HPLC) on a C4 medium, and the single-step elution of purified hormone from concanavalin A agarose. Overall, an 890-fold purification was accomplished with a recovery of 80% as assayed in vitro. Biologically, this purified erythropoietin is highly active, possessing a specific activity in vitro of 200,000 U/mg protein. Chemically, this erythropoietin (molecular weight [mol wt] 26,200) appears exceptionally uniform in its oligosaccharide constitution (30%) as contrasted with heterogeneously glycosylated erythropoietins derived from mammalian cells (mol wt 30,000 to 38,000; 40% to 50% complex-type oligosaccharide). Thus, human erythropoietin as presently produced in an insect cell line comprises not only an abundant source of highly active, readily purified hormone for studies of its mechanism of action and cell surface receptor, but also represents a uniquely homogeneous form that should prove advantageous for direct structural analyses.

ERYTHROPOIETIN is a prime regulator of RBC production in mammals and birds. Specifically, this glycoprotein hormone promotes the rapid growth of red cell progenitors in marrow, spleen, and fetal liver, and subsequently is required for their terminal differentiation to circulating erythrocytes. Historically, studies of this factor have been limited by its low availability with urine from select patients with aplastic anemia comprising the most abundant natural source. Recently, the availability of erythropoietin has been improved through the isolation of the human erythropoietin gene and cDNA, and the production of active recombinant hormone using mammalian expression systems. We previously have developed a recombinant baculovirus vector that mediates the production of human erythropoietin in cultured insect cells. In contrast to human erythropoietins which, as expressed in mammalian cells, contain substantial amounts of heterogeneous, complex-type oligosaccharides (molecular weight [mol wt] 35,000), this recombinant human erythropoietin (mol wt 26,200) apparently is glycosylated uniformly with N-linked oligosaccharides of only limited size, yet possesses high biological activity as assayed in vitro. Based on its distinct and relatively simple physicochemistry, we now have established conditions for expression of this erythropoietin at high levels, as well as a simple and efficient procedure for its purification to homogeneity. Thus, this erythropoietin is highly suitable for studies of its cell surface receptor and presently unresolved mechanism of action. In addition, its uniquely simple and homogeneous chemistry should prove advantageous in direct structural analyses.

MATERIALS AND METHODS

Cell culture and expression of recombinant erythropoietin. Spodoptera frugiperda cells (ATCC) were maintained at 28°C in TMN-FH medium, 10% fetal calf serum (FCS). For the expression of recombinant erythropoietin, cells were grown in spinner flasks to a density of 10⁶ cells/mL, collected by centrifugation, and incubated at 10°C for 1 h with clone recombinant baculovirus. Following subsequent culture for three days in Grace's medium, 4% FCS, 20% TMN-FH, spent medium was collected, concentrated 15-fold (tangential flow system, 10,000 mol wt PLGC filter) and dialyzed against 1 mM Na2EDTA, 10 mMol/L PIPES, pH 6.8.

Purification of erythropoietin. Concentrated media initially was passed over a column of DEAE-Sephacel (Pharmacia, Piscataway, NJ) (1 mL gel/mL dialyzed media) under isocratic conditions in 0.02% octyl-beta-D-glucopyranoside, 10 mMol/L K2PIPES, pH 6.8. Column eluate and two column volumes of buffer wash were collected, pooled, and concentrated 40-fold. Concentrate then was subjected directly to reverse-phase high performance liquid chromatography (HPLC) on a C4 medium (Supelcosil LC-304, 25 cm x 1 cm column). Samples were applied in 0.06% trifluoroacetic acid as an initial mobile phase, and were chromatographed using a linear gradient of acetonitrile (30% to 50%) in 0.06% trifluoroacetic acid. Eluted fractions were collected into vials containing 5 μL of 1% octyl-beta-D-glucopyranoside, and were dried under vacuum. HPLC fractions that were shown by bioassay and by Western blot analyses to contain erythropoietin were resolubilized in 1 mol/L NaCl, 1 mmol/L MgCl2, 1 mmol/L MnCl2, 1 mmol/L CaCl2, 0.02% octyl-beta-D-glucopyranoside, 10 mMol/L PIPES pH 6.8, and were applied to a column of concanavalin A agarose (Sigma Chemical Co, St Louis) (4 cm column). The column was washed with 100 μL of the above buffer, and erythropoietin was eluted in buffer containing 250 mMol/L alpha-methyl-D-mannoside. Subsequently, this eluate was dialyzed at 4°C against 0.01% octyl-beta-D-glucopyranoside.

Biologic assay of erythropoietin. The biologic activity of various preparations was determined by quantifying the erythropoietin-dependent incorporation of [3H]thymidine into splenocytes derived from mice.
from mice treated with phenylhydrazine.7 Assays were standardized against the World Health Organization Second International Reference Preparation, calibrated recombinant human erythropoietin standards, and purified recombinant human erythropoietin produced in CHO cells (160,000 U/A280 Amgen, Thousand Oaks, CA). The activity of purified hormone also was assayed in vitro using a CFU-e colony forming assay13 and in vivo in ex-hypoxic, polycythemic mice.14

**Electrophoresis and protein assay.** Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli19 in 12% gels. In specified instances, laser densitometry of silver-stained gels was used to verify purification factors and recoveries. Western blot analyses were performed essentially as described by Towbin20 using antibodies prepared against recombinant human erythropoietin produced in CHO cells. Protein assays were performed using BCA reagent (Pierce Chemical, Rockford, IL) and chromatographically purified BSA (Cappel, Malvern, PA) as a standard.

**RESULTS**

**Expression of recombinant erythropoietin by S frugiperda cells.** In experiments designed to maximize the expression of recombinant erythropoietin in media with reduced protein content, the effects of culturing infected S frugiperda cells in limiting amounts of FCS and media supplements were assessed. Expression in media containing FCS at concentrations of <4% was diminished substantially. Also, erythropoietin produced under these conditions was composed of a mol wt 26,000 form, plus two alternate forms of lower molecular weight (mol wt 24,000 and 22,000). As shown below, this was established in part by Western blot analyses using antibodies prepared against purified human erythropoietin. In contrast, a singular form of recombinant erythropoietin (mol wt 26,000) was expressed by S frugiperda cells cultured in media containing ≥4% FCS. Also, a significant increase in levels of secreted erythropoietin (ie, 500,000 U/L at 72 hours postinfection; Table 1) was achieved by supplementing media with yeast and lactalbumin hydrolysates at 20% of the nominal levels used in routine cell culture. Evidence that secretion proceeded efficiently under these conditions was given by the lack of detectable amounts of immunoreactive erythropoietin in cell lysates.

**Purification of recombinant human erythropoietin.** The purification of recombinant human erythropoietin to virtual homogeneity was accomplished via three chromatographic steps. First, erythropoietin-containing media (500,000 U/L, Table 1) was concentrated, dialyzed, and passed over a column of DEAE-Sepharose under isotonic conditions (0.02% octyl-beta- d-glucopyranoside, 10 mmol/L K2-PIPES, pH 6.8). Erythropoietin eluted directly with high recovery (>95% of initial activity), while approximately 98% of the total protein contained in dialyzed media was retained. Thus, this simple procedure provides an initial 45-fold purification.

Material recovered following chromatography on DEAE-Sepharose was concentrated, and was chromatographed directly on a reverse-phase C4 medium. As established by bioassay, erythropoietin eluted in 44% acetonitrile, with an estimated recovery of >95% of active hormone (Fig 1). No biologically or immunologically active hormone was detected in any other portion of the elution profile. Routinely, the injection of 60,000 U to 100,000 U per run resulted in a similar elution pattern and recovery of hormone (Table 1). Analysis of this material by SDS-PAGE showed that several contaminating proteins co-eluted from C4 medium with erythropoietin, which was estimated by densitometry of silver-stained gels to represent 15% of total protein. By comparison, erythropoietin represented 5% of total protein before C4 HPLC. Thus, this step provided a threefold purification. A sixfold further purification of erythropoietin was accomplished simply by reconstituting this material in buffer containing NaCl at a high molarity (ie, ≥1 mol/L). Under these conditions the majority of contaminating proteins were insoluble (approximately 85% of total protein) and were removed readily by brief microcentrifugation. In contrast, erythropoietin was fully soluble at concentrations as high as 200,000 U/mL as was established by its reconstitution at increasing concentrations, and subsequent biologi-

**Table 1. Purification of Recombinant Human Erythropoietin**

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Units Processed per Step</th>
<th>Recovery</th>
<th>Purification Overall</th>
<th>Specific Activity</th>
</tr>
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<tr>
<td>Media</td>
<td>500,000</td>
<td>—</td>
<td>—</td>
<td>230 U/mg</td>
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<tr>
<td>Concentration/DEAE</td>
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<td>810</td>
<td>180,000 U/mg*</td>
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<td>Concanavalin A</td>
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<td>&gt;95%</td>
<td>890</td>
<td>200,000 U/mg</td>
</tr>
</tbody>
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*Estimated via laser densitometric analysis before and after C4 chromatography. Otherwise, recoveries and specific activities were measured by direct protein and biological assays.

![Fig 1. Reverse-phase chromatography of erythropoietin. Following DEAE chromatography, erythropoietin (350 U in 0.1 mL) was applied to a reverse-phase (C4) HPLC column, and was developed with a linear gradient of acetonitrile (3.3% acetonitrile/min, 1 mL/min flow rate). The elution of protein was monitored by absorbance at 254 nm (----). The concentration of erythropoietin in various fractions (0.2 mL) was determined by biological assay (- - -).](http://www.bloodjournal.org/doi/abs/10.1182/blood.87.1.653)
Fig 2. Analysis of purified erythropoietin by SDS-PAGE. The purity of erythropoietin as isolated by chromatography on DEAE-Sephacel, C4 medium, and concanavalin A agarose was assessed by SDS-PAGE (12% gel, reducing conditions) and silver-staining. Scanning laser densitometry (right panel) was used to confirm purity (>99%). Molecular weight standards (mol wt x 10^3) are indexed in the left margin.

Fig 3. Isolation of mol wt 26,000 and 24,000 erythropoietins and comparison of specific bioactivities. Erythropoietin expressed under low serum conditions (1% FCS) and purified via reverse-phase HPLC (approximately 90% purity) was subjected to SDS-PAGE in the absence of 2-mercaptoethanol. Alternate forms were visualized by Western blotting (A, lane A). A parallel gel lane was sectioned into 1 mm slices, and erythropoietins then were eluted into 0.5 ml of phosphate-buffered saline containing 0.02% Tween-20 and 0.1 mg/ml BSA. Eluates then were subjected to biological assay (B), and to Western blot analysis (A) in order to confirm recoveries and separation.
Fig 4. Analysis of N-glycosylation of mol wt 28,000 and 24,000 erythropoietins using N-glycanase. Purified erythropoietins (350 U) expressed under low serum conditions (1% FCS) were incubated with N-glycanase (3 U) in 0.25% SDS, 0.25 mol/L phosphate pH 8.6, at 37°C for 18 hours. Products (+) were analyzed by SDS-PAGE in the absence of 2-mercaptoethanol, and were visualized by silver-staining. A control sample (−) was processed in the same manner in the absence of N-glycanase.

weight of aglycosyl apo-erythropoietin (mol wt 18,400) as determined by SDS-PAGE (Fig 4), thus indicating that each form is N-glycosylated, and is not processed proteolytically beyond its secreted form. However, a minor residual difference in the electrophoretic mobilities of these endoglycolytic products (mol wt 18,900 v 18,400) was detectable. The basis for this difference presently is unresolved. The third form of erythropoietin (mol wt 22,000, Fig 4, lane A) was found to bind poorly, if at all, to concanavalin A agarose. Due to its low abundance, the specific biological activity of this form has not been estimated directly. However, based on the above observations, this erythropoietin is believed to possess a third distinct oligosaccharide constitution.

In order to provide independent estimates of molecular weight and relative saccharide content, the Stokes’s radius (2.56 nm) and sedimentation coefficient (2.48 x 10⁻¹³ seconds) of the purified mol wt 26,000 form were determined (Fig 5, Table 2). A molecular weight of 26,200 is predicted from these values. Thus, the estimate of relative saccharide content (30%) is in approximate accordance with estimates based on SDS-PAGE.

Fig 5. Determination of Stoke’s radius and sedimentation coefficient of human erythropoietin produced in S. frugiperda cells. The Stoke’s radius (A) of insect erythropoietin was evaluated by HPLC gel filtration chromatography (G2000 SWXL 30 cm x 0.78 cm column, Supelco). Column calibration was performed using DNA (Sigma type III), bovine serum albumin, ovalbumin, and cytochrome C standards (O—O). The elution of standards was monitored by absorbance at 264 nm. The elution position of erythropoietin (●); 2.56 nm Stokes radius) was confirmed by Western blot analysis. Evaluation of the sedimentation coefficient (2.48 x 10⁻¹³ sec; B) was estimated by rate sedimentation in a 5% to 20% sucrose density gradient. Purified erythropoietin (●) was sedimented with standards (O—O; bovine serum albumin and cytochrome C) (18 hours at 40,000 rpm in a SW50.1 rotor). Fractions (0.16 mL) were collected and analyzed by SDS-PAGE.

DISCUSSION

Systems previously developed for the production of recombinant human erythropoietin have depended on the use of mammalian expression systems and a variety of cell lines including COS cells, baby hamster kidney cells, Chinese hamster ovary cells, and psi-2 cells. As is the case with urinary erythropoietin, recombinant hormone produced in mammalian cells is heterogeneously glycosylated, with complex N- and O-linked oligosaccharides comprising as much as 50% of total molecular mass. In contrast, human erythropoietin as presently prepared in S. frugiperda cells is exceptional in both its comparably simple saccharide constitution, and its associated relative homogeneity. In general, the limited capacity of insect cells to process N-linked oligosaccharides to complex types results in a predominance of neutral oligosaccharides of the high mannose type. The prospect that at least certain oligosaccharides of the human erythropoietin presently prepared are of this type is sup-
ported by its comparably low saccharide content (approximately 30%) and reactivity with concanavalin A (Table 1). The apparent high uniformity among molecules of this mol wt 26,000 erythropoietin (Fig 2) is evidenced further by the indication that two alternate forms (mol wt 24,000 and 22,000) produced by S frugiperda cells cultured in low serum correspond to alternatively glycosylated products. This conclusion is based first on the observation that treatment of the mol wt 26,000 and 24,000 forms with N-glycanase yields erythropoietin peptides of nearly equivalent molecular weight (ie, 18,900 v 18,400). Second, the failure to detect erythropoietin peptides of lower molecular weight by Western blot analysis argues against any possible nonspecific proteolysis of secreted products. Third, the occurrence in erythropoietin of three consensus sites for N-linked glycosylation correlates well with the observed occurrence of three forms of glycopeptide. Thus, these different forms may derive from the differential utilization of glycosylation sites, with the mol wt 26,000 form glycosylated uniformly at each site. Importantly, the high specific in vitro biological activity possessed by this purified erythropoietin confirms that this relatively limited level of glycosylation is sufficient for its normal interaction with the cell surface receptor of mammalian erythroid cells. The absence of sialic acid in insect-derived saccharide structures is a likely explanation for the lack of in vivo activity.

Previously reported procedures for the purification of human recombinant erythropoietins generally have been based on methods developed by Miyake et al for the isolation of human urinary erythropoietin. Commonly, these procedures involve the use of five or more separate conventional chromatographic steps. Alternatively, the isolation of recombinant hormone has been simplified somewhat by the development of an efficient immunosorbant. However, this approach requires the use of a proprietary monoclonal antibody, the denaturation of erythropoietin with SDS for efficient epitope recognition, and at least two additional conventional chromatographic steps. In contrast, the procedure described here achieves >99% purity via three simple chromatographic steps (Table 1). An 890-fold purification is accomplished with ≥80% recovery of bioactive erythropoietin. While this manuscript was in preparation, a procedure for the purification of recombinant human erythropoietin expressed in BHK cells was reported, which involves three distinct chromatographic procedures, ie, sequential chromatography on CM Affi-Gel blue, Mono Q (FPLC), and a C18 reverse-phase medium. This procedure resulted in a 160-fold purification yielding a product with a specific activity of 84,000 U/mg. In contrast to the present procedure, gradient elution of products was required at each step. In addition, the overall reported recovery of this product was substantially lower (ie, 35%), and its final purity was not fully assessed.

The baculovirus-mediated expression system presently developed for the production of recombinant human erythropoietin provides a convenient source of milligram amounts of highly active growth factor. Whereas the simple glycochemical properties of this erythropoietin have facilitated its purification to virtual homogeneity, it is anticipated that this unique human erythropoietin also should facilitate direct structural analyses aimed at identifying physicochemical and structural features essential to its bioactivity.

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