Detection and Isolation of the Erythropoietin Receptor Using Biotinylated Erythropoietin

By Albertus W. Wognum, Peter M. Lansdorp, R. Keith Humphries, and Gerald Krystal

Procedures have been developed to label human erythropoietin (Ep) with biotin to detect and isolate the Ep-receptor. The labeling method used the abundant carbohydrate groups on Ep and resulted in biologically active biotin-Ep (b-Ep) containing 8 to 10 biotins per Ep molecule. Specific binding of b-Ep to cells from spleens of mice made anemic by phenylhydrazine injections was demonstrated using 125I-labeled streptavidin. b-Ep, together with fluorescently tagged streptavidin, was found to specifically detect Ep-receptor-bearing cells by flow cytometry. This was demonstrated in several ways. First, approximately 90% of nucleated spleen cells from phenylhydrazine-treated mice were clearly fluorescent after staining with b-Ep and streptavidin-phycocerythrin, whereas only background fluorescence was detected using spleen cells from untreated mice. In addition, Ep-receptors were detected on 5% to 10% of normal mouse bone marrow cells, and these cells could be identified as erythroid in nature by separating the cells into subpopulations based on light-scatter properties. Third, Ep-receptor expression was found to correlate positively with expression of transferrin receptors, confirming the erythroid nature of these cells. b-Ep was also used to isolate Ep-receptors from monkey COS cells transfected with the murine Ep-receptor cDNA. In these experiments a cell-surface-bound protein of ~65 Kd and an intracellular protein of ~60 Kd were isolated from these cells. The procedures described in this report for detecting Ep-receptor expressing cells and for isolating the Ep-receptor should be valuable for purifying erythroid cells from heterogeneous cell populations, for elucidating the structure of the Ep-receptor, and for studying the biological activities of Ep at the cellular and molecular level.

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underderivatized Ep (420 U/mL) were diluted fivefold in RPMI containing 20% fetal calf serum (FCS) and 10^{-4} mol/L 8-mercapto-ethanol. One hundred fifty microliters of these preparations (corresponding to 6 and 18 pmol of Ep, respectively) were added to 50 μL of a suspension of streptavidin-agarose (Catalog no. 2034, Pierce, Rockford, IL) corresponding to 25 to 50 pg (0.4 to 0.8 nmol) streptavidin and incubated for 2.5 hours at 4°C. After removal of the beads by centrifugation, the biologic activity remaining in the supernatant was measured using the mouse spleen-cell assay.

Molecular weight determination. Biotinylated Ep and underivatized Ep were labeled with 125I using the chloramine T method. The 125I-labeled preparations, together with 14C-labeled mol wt markers (Amersham), were then analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE). The dried gels were exposed to Kodak X-Omat AR film for 2 days at -70°C.

Cell preparation. Adult C3H/He x C57B1/6j (C3B6F1) mice were used in two injections with phenylhydrazine, as described. After 5 days the enlarged spleens were removed and single-cell suspensions prepared. Mature RBCs were removed by osmotic lysis in 10 mmol/L potassium bicarbonate, 155 mmol/L sodium chloride buffer, pH 7.4, containing 0.1% (wt/vol) EDTA.

The nucleated cells were washed twice with Hanks balanced salt solution, containing 1% (wt/vol) bovine serum albumin (BSA) and 0.05% (wt/vol) sodium azide (HHBA-buffer) and resuspended in the same buffer. Bone marrow cells were obtained from the hind limbs of untreated adult C3B6F1 mice, and nucleated cells were prepared as described for the spleen cells. In some experiments nucleated bone marrow cells were isolated by density separation using Ficoll-Paque (Pharmacia), and cells were washed with and resuspended in Hanks balanced salt solution containing BSA, without sodium azide.

Binding analysis. Specific binding of b-Ep to murine erythroid progenitor cells was measured using a two-step procedure. In the first step 3 × 10⁸ erythroid progenitor cells in a volume of 200 μL HHBA buffer were incubated with b-Ep (1 nmol/L) or, as controls, with buffer only or with b-Ep plus a 100-fold excess of unlabeled Ep. After optimal binding was achieved (3 hours at 20°C), the cells were washed twice with ice-cold HHBA to remove unbound ligand. In the second step cells were incubated with streptavidin (Sigma, St Louis, MO), that was labeled with 125I to a specific activity of 14 × 10⁶ cpm/μg (8.4 × 10⁴ cpm/mmol) using the iodogen method. Each tube received 100,000 cpm of 125I-streptavidin, yielding a streptavidin concentration of 1.2 nmol/L, and incubation was continued for 0.5 to 1 hour at 4°C. The cell suspensions were then transferred to 0.5 ml microfuge tubes containing 200 μL of a dibutyl phthalate/ dibutyl phthalate oil mixture (3/2 ratio) and centrifuged for 3 minutes at 14,000 rpm in an Eppendorf microfuge to separate cells from unbound radioactivity. The tubes were frozen and the tips containing the cell pellets were cut off, transferred to γ-tubes, and counted in a Beckman 5500 γ-counter.

### Table 2. Biotin Labeling of Carbohydrate Groups on Ep: Procedure and Recovery of Biologic Activity

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Ep-Titer (U/mL)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mild periodate oxidation of sialic acid residues on Ep: removal of excess periodate by gel filtration.</td>
<td>770</td>
<td>77</td>
</tr>
<tr>
<td>Control (no oxidation)</td>
<td>896</td>
<td>100</td>
</tr>
<tr>
<td>Conjugation of aldehyde groups on Ep with biotin-amino-caproylhydrazide: removal of excess reagent by gel filtration.</td>
<td>350</td>
<td>39</td>
</tr>
<tr>
<td>Control</td>
<td>1,008</td>
<td>112</td>
</tr>
</tbody>
</table>

Fluorescence staining of erythroid cells. To measure Ep-receptor expression, 2.5 × 10⁸ spleen cells from phenylhydrazine-treated or untreated mice or 1 × 10⁶ normal nucleated mouse bone-marrow cells, in a volume of 300 μL, were incubated with b-Ep (1 nmol/L) or, as controls, with buffer only or with b-Ep in the presence of excess unlabeled Ep. In preliminary experiments, incubations were performed for 3 hours at 20°C using HHBA buffer containing 0.05% sodium azide, but this was later shortened to 1 hour at 37°C, since a similar level of fluorescence was obtained at this temperature, even in the absence of azide. After incubation, cells were washed twice with ice-cold HHBA and incubated with a streptavidin-R phycoerythrin (RPE) conjugate (Molecular Probes, Eugene, OR). This incubation was typically performed for 1 hour at 4°C, but identical results were obtained at 20°C. After incubation, cells were washed and analyzed on a flow cytometer. For double-staining experiments, human transferrin (Sigma) was first saturated with iron to increase its affinity for its cell-surface receptor and then conjugated to fluorescein isothiocyanate (FITC) using standard procedures. Transferrin-FITC was added at a final concentration of 10 μg/mL during the incubation step with streptavidin-RPE. As a control, cells were also stained with transferrin-FITC alone.

Flow cytometry. Quantitative fluorescence analyses were performed using FACStar plus and FACScan flow cytometers (Becton-Dickinson, Sunnyvale, CA). For two-color experiments, spillover of green fluorescence into the red fluorescence detector was electronically compensated to background levels using cells stained only with transferrin-FITC (green). For most experiments 50,000 events were collected. Analysis of Ep-receptor expression on populations of cells identified by forward- and right-angle light scatter properties or by green fluorescence intensity after staining with transferrin-FITC was performed using Consort-30 and Lysis data-management software (Becton-Dickinson), respectively. In double-staining experiments an electronic window was set for forward- and right-angle light scatter to exclude granulocytes from the analysis. This facilitated analysis of double-positive cells by gating out cells that exhibited strong green and red autofluorescence. Contour plots of forward-angle versus right-angle light scatter were drawn at 9 levels, with an initial level of 5 and at increments of 10 cells per level.

Transfection of COS-1 cells, metabolic labeling, and affinity isolation of the Ep-receptor. Subconfluent monolayers of COS-1 cells, grown in 13-cm tissue culture dishes (Becton-Dickinson), were transfected with 12 μg of plasmids containing either clone 141 or clone 190 of the cDNA encoding the murine Ep-receptor. Both plasmids were generously provided by Dr A.D. D’Andrea (Whitehead Institute, Cambridge, MA). As a control, cells were also transfected with the plasmid minus the insert. Transfections were performed using the diethyl aminoethyl (DEAE)-dextran method.

Table 2. Adsorption of b-Ep by Streptavidin-Agarose

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Ep-Titer (U/mL)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Underivatized Ep</td>
<td>80</td>
<td>100</td>
</tr>
<tr>
<td>Underivatized Ep after exposure to streptavidin-agarose</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>Biotin-Ep</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>Biotin-Ep after exposure to streptavidin-agarose</td>
<td>0.046</td>
<td></td>
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DETECTION OF EP RECEPTORS WITH BIOTIN-Ep

Fig 1. Molecular weight determination of biotinylated erythropoietin. Biotinylated urinary Ep (biotin-Ep no. 1), biotinylated recombinant Ep (biotin-Ep no. 2), and nonderivatized (urinary) Ep were labeled with ^125I, electrophoresed on a 10% SDS-polyacrylamide gel, and the fixed and dried gel subjected to autoradiography. Molecular weights of coelectrophoresed standards are indicated in the margin.

Biotin labeling of Ep. Preliminary attempts to biotinylate Ep using aminoreactive reagents resulted in the irreversible loss of Ep's biological activity (data not shown). We therefore investigated whether sialic acid residues on Ep

were chilled on ice and 0.5 mL of a 40 mmol/L solution of 3[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS; Sigma) added to a final concentration of 8 mmol/L to solubilize membrane proteins. In some experiments cells were washed twice with HHBA-buffer containing protease inhibitors prior to solubilization. After 1 hour incubation at 4°C, insoluble material was removed by microfuging for 10 minutes at 14,000 rpm. The supernatants containing solubilized membrane proteins were first preclarified with 60 mg glycine-blocked Sepharose-4B beads (Pharmacia, Uppsala, Sweden) for 1 hour at 4°C and then incubated for 2 hours at 4°C with 10 mg streptavidin-agarose beads to specifically adsorb b-Ep/receptor complexes. The beads were exhaustively washed with HHBS-buffer containing 8 mmol/L CHAPS, 0.5 mmol/L PMSF, 100 U/mL aprotinin, and 40 µg/mL leupeptin and bound proteins eluted by boiling for 3 minutes with SDS-sample buffer, containing 5% (vol/vol) β-mercaptoethanol, and analyzed by SDS-PAGE. After electrophoresis the fixed gels were treated with En'HANCE (NEN Research Products, Boston, MA) and exposed to Kodak X-Omat film for 7 days at −70°C.

RESULTS

Biotin labeling of Ep. Preliminary attempts to biotinylate Ep using aminoreactive reagents resulted in the irreversible loss of Ep's biological activity (data not shown). We therefore investigated whether sialic acid residues on Ep

Fig 2. Binding of biotinylated Ep to erythroid precursor cells. Spleen cells from phenylhydrazine-treated mice (10^7) were incubated with b-Ep (1 nmol/L alone, or in the presence of 100 nmol/L unlabeled Ep or 25 µg/mL of the MoAbs anti-Ep-2, -16, -26, or a control antibody (antialkaline phosphatase), respectively. After 3 hours at 20°C, cells were washed and incubated for 1 hour with ^125I-streptavidin, and the amount of bound radioactivity was measured. Results are expressed as mean ± SE of triplicates. Binding of ^125I-streptavidin to cells incubated without b-Ep was 590 ± 40 cpm.
with b-Ep in the presence of an 100-fold molar excess of unlabeled Ep were incubated with streptavidin-agarose beads and the activity was due to underivatized Ep molecules in the b-Ep preparation, biotin-labeled Ep as well as underivatized Ep completely lost by adsorption to immobilized streptavidin, suggesting that some b-Ep molecules were biologically active. To exclude the possibility that the biological activity of the b-Ep preparation was almost completely lost by adsorption to immobilized streptavidin, demonstrating that b-Ep could bind specifically to the Ep-receptor on the surface of normal erythroid progenitor cells and could be detected by virtue of the high-affinity interaction of biotin with radiolabeled streptavidin. As is also shown in Fig 2, three monoclonal antibodies (MoAbs) against Ep that we had developed previously were also capable of inhibiting the specific binding of Ep to these cells, whereas a control antibody (anti-alkaline phosphatase) had no effect. The inhibitory activity of the anti-Ep MoAbs shown in Fig 2 correlated well with their ability to neutralize the biological activity of Ep in vitro, demonstrating that their neutralizing activity was caused by the inability of Ep/anti-Ep immune complexes to bind to cell-surface receptors.

Specific binding of b-Ep to erythroid progenitor cells. To test the ability of b-Ep to bind specifically to its cell-surface receptor on erythroid precursor cells, a two-step binding assay was performed. In this procedure, murine erythroid precursor cells, isolated from the regenerating spleens of mice made anemic by two injections with phenylhydrazine, were first incubated with b-Ep and then with 125I-streptavidin. As shown in Fig 2 (first column), incubation with b-Ep (1 nmol/L) resulted in a total binding of approximately 2600 cpm of 125I-streptavidin to 1 × 10⁸ spleen cells. However, in the presence of a 100-fold excess of unlabeled Ep, only 750 cpm were bound. These results demonstrated that b-Ep could bind specifically to the Ep-receptor on the surface of normal erythroid progenitor cells and could be detected by virtue of the high-affinity interaction of biotin with radiolabeled streptavidin. As is also shown in Fig 2, three monoclonal antibodies (MoAbs) against Ep that we had developed previously were also capable of inhibiting the specific binding of Ep to these cells, whereas a control antibody (anti-alkaline phosphatase) had no effect. The inhibitory activity of the anti-Ep MoAbs shown in Fig 2 correlated well with their ability to neutralize the biological activity of Ep in vitro, demonstrating that their neutralizing activity was caused by the inability of Ep/anti-Ep immune complexes to bind to cell-surface receptors.

Detection of Ep-receptor expressing cells by flow cytometry. B-Ep was tested for its usefulness in detecting erythroid progenitor cells in heterogeneous cell populations. For this purpose spleen cells from phenylhydrazine-treated mice were labeled, first with b-Ep and then with streptavidin-RPE. The cells were then analyzed by flow cytometry. As shown in Fig 3A, a large fraction of the cells labeled with b-Ep could be used as attachment sites for biotin, since these sugar moieties have been shown to be unnecessary for biological activity. Specifically sialic acid residues on Ep were mildly oxidized with periodate and then conjugated with biotin-hydrazide. As shown in Table 1, a significant proportion of Ep's biological activity (ie, 39%, [assayed using the mouse spleen-cell assay]) was still present after completion of the procedure, suggesting that some b-Ep molecules were biologically active. To exclude the possibility that the biological activity was due to underivatized Ep molecules in the b-Ep preparation, biotin-labeled Ep as well as underivatized Ep were incubated with streptavidin-agarose beads and the biological activity remaining in the supernatants assayed. As shown in Table 2, all of the underivatized Ep in the control preparation was recovered after this procedure. In contrast, the biological activity of the b-Ep preparation was almost completely lost by adsorption to immobilized streptavidin, demonstrating that all the biologically active Ep molecules in the b-Ep preparation were indeed labeled with biotin.

Further analysis of two separate preparations of b-Ep by SDS-PAGE demonstrated that the conjugation with biotin resulted in a 3,000 to 4,000 dalton increase in apparent mol wt (Fig 1). Since biotin-hydrazide has a mol wt of 370 daltons, this increase corresponds to an estimated 8 to 10 biotin molecules per Ep-molecule in both preparations. The minor 68-Kd protein observed in b-Ep preparation no. 2 (Fig 1) may correspond to a contamination with albumin or to a small amount of dimerized Ep.
b-Ep and streptavidin-RPE were clearly fluorescent, whereas cells that had been labeled with streptavidin-RPE alone or with b-Ep in the presence of a 100-fold molar excess of unlabeled Ep showed only background fluorescence. In contrast, no Ep-receptor–positive cells were observed when spleen cells from untreated mice were analyzed (Fig 3B), consistent with the low number of Ep-responsive cells present in normal mouse spleens (G. Krystal, unpublished results, January 1983).

Normal mouse bone marrow was also analyzed for Ep-receptor–positive cells, and approximately 5% to 10% of these cells were clearly fluorescent, whereas cells labeled with either b-Ep in the presence of an excess of unlabeled Ep or with streptavidin-RPE alone showed only background fluorescence (Fig 3C). To further characterize these Ep-receptor–positive cells, four subpopulations of normal nucleated mouse bone marrow cells (ie, erythroid cells, lymphoid cells, granulocytes, and blast cells) were selected on the basis of their forward- and right-angle light scatter properties, and Ep-receptor expression was analyzed within each subpopulation (Figs 4 and 5). The stained cells were analyzed either ungated (Fig 5A) or in electronic windows for forward- and right-angle light scatter indicated by the boxes in Fig 4 (Fig 5B through F). The resulting fluorescence profiles indicated that granulocytes, late myeloid precursor cells, and monocytes present in the “G” window of Fig 4 did not express detectable numbers of Ep-receptors (Fig 5B). In contrast, most cells in the “E” window of Fig 4 were found to be Ep-receptor positive (Fig 5C). These cells represented ~10% of the total cell population, and analysis of sorted cells indicated that they consisted mainly of normoblasts and ~5% reticulocytes. A small subpopulation of Ep-receptor–positive cells was also identified when cells were analyzed in the “L” window, suggesting that this window may contain not only lymphoid cells but cells of erythroid origin as well. The majority of cells in this window with relatively low side-scatter properties (Fig 4, part b of “L” window) showed a fluorescence profile that was similar to that of cells in the total “L” window (Fig 5E). However, a small fraction of cells with relatively high side-scatter properties (Fig 4, part a of “L” window) and representing ~2% of all nucleated bone marrow cells consisted of more than 80% Ep-receptor–positive cells (Fig 5D). Analysis of the morphology of cells...

Fig 6. Correlation between transferrin-receptor and Ep-receptor expression on normal mouse bone marrow cells. Cells were stained as described in the legend to Fig 3, except that they were incubated at the same time with both streptavidin-RPE and transferrin-FITC. (A) Green fluorescence of cells stained with (—) or without (-----) transferrin-FITC. The arrows in A indicate the transferrin-receptor low (fraction I) and high cells (fraction II) selected for Ep-receptor expression analysis. (B through D) Red fluorescence was analyzed for all cells (B), for cells in fraction II (C), and for cells in fraction I (D). Fluorescence profiles in B, C, and D are of cells stained with b-Ep (——), streptavidin-RPE alone (-----), or b-Ep plus excess unlabeled Ep (-----). Ep-receptor–negative granulocytes were excluded from the analysis by selecting a window for cells with low right-angle light-scatter properties.

Fig 5. Fluorescence profiles of mouse bone marrow cells stained for Ep-receptor expression. Normal mouse bone marrow cells were incubated with b-Ep (——) or, as controls, with buffer only (-----) or with b-Ep in the presence of a 100-fold molar excess of unlabeled Ep (-----). After 1 hour incubation at 37°C, cells were washed, incubated for 1 hour at 4°C with streptavidin-RPE, washed again, and analyzed on a flow cytometer. Fluorescence was analyzed for all cells (A) or for cells gated in the windows for forward- and right-angle light scatter indicated by the boxes in Fig 2 (B through F).
sorted in this window suggested that they consisted mainly of proerythroblasts and thus represented a more primitive and less abundant population of erythroid progenitor cells than those identified in the "E" window. As shown in Fig 5F, a small subpopulation of Ep-receptor-positive cells (less than 1% of total cells) was also identified in the blast cell window, which contains CFU-S and other primitive hematopoietic cells.3

Double-staining experiments in which cells were stained with both biotin-Ep/streptavidin-RPE and transferrin-FITC showed a strong correlation of Ep-receptor and transferrin-receptor expression, the latter known to be strongly expressed on differentiating erythroid cells because of their high iron requirements.24 As shown in Fig 6A, the majority of nucleated bone marrow cells was transferrin-receptor positive, as indicated by the shift of the fluorescence profile with respect to that of cells not stained with transferrin-FITC. When the fraction of cells that was stained most strongly by transferrin-FITC (Fraction II, Fig 6A) was analyzed for Ep-receptor expression, a large subpopulation of Ep-receptor-positive cells, representing more than 50% of the cells, was identified (Fig 6C). In contrast, only background fluorescence was observed when the cells that had low transferrin-receptor expression (fraction I, Fig 6A) were analyzed for Ep-receptor expression (Fig 6D). For comparison, the b-Ep/streptavidin-RPE fluorescence profile of un gated cells is shown in Fig 6B. Taken together these results indicate that Ep-receptor expression correlates positively with expression of the transferrin-receptor on mouse bone marrow cells, demonstrating that b-Ep, in combination with fluorescein tetramethyl rhodamine isothiocyanate (FITC) labeled streptavidin, can specifically detect erythroid cells.

Affinity isolation of the Ep-receptor from transfected COS cells. The Ep-receptor has been reported to consist of two proteins of approximately 100 and 85 Kd, as determined by chemical cross-linking of 125I-Ep to the surface of erythroid cells,25 and both proteins appear to be products of a single gene encoding a 55-Kd polypeptide.18 To examine the relationship between the two proteins and to characterize the Ep-receptor at the protein level, we isolated the Ep receptor using b-Ep and immobilized streptavidin. Specifically, monkey COS cells transfected 2 days previously with either clone 141 or clone 190 of the cloned murine Ep-receptor cDNA18 and expressing, on average, 55,000 Ep receptors per cell, were metabolically labeled with 35S-labeled amino acids. The radiolabeled cells were then incubated with b-Ep or, as controls, with buffer only or with b-Ep in the presence of an...
DETECTION OF Ep RECEPTORS WITH BIOTIN-Ep

excess of unlabeled Ep. After washing away unbound b-Ep, plasma membrane proteins were then extracted by solubilization with CHAPS under conditions that preserve the interaction between Ep and its receptor. Solubilized b-Ep/receptor complexes were then specifically adsorbed to streptavidin-agarose beads and, after washing, eluted from the beads by boiling in SDS-sample buffer and analyzed by SDS-PAGE. Streptavidin-agarose bound a ~65-Kd protein from the extracts of cells transfected with either clone 141 (Fig 7, lane 2) or clone 190 (Fig 7, lane 5) and incubated with b-Ep. Several other bands were also visible in these lanes, but these represented proteins that had nonspecifically bound to the streptavidin-agarose, since they were also present in the control lanes. In addition, the 65-Kd band was not detected when cells transfected with an irrelevant plasmid were used (lanes 7 through 9). When unbound b-Ep was not washed away before solubilizing the cells, a minor 60-Kd protein was also detected (Fig 8). Similar results were obtained when b-Ep was added only after solubilization of the cells (data not shown), and these results suggest that the 65-Kd protein is expressed on the cell surface, whereas the 60-Kd protein is present only intracellularly.

DISCUSSION

In this study we demonstrate that human Ep can be labeled on sialic acid moieties with the hapten biotin with good retention of biological activity. Since the carbohydrate on Ep is not essential for receptor binding, this approach is preferable to more conventional labeling procedures that involve derivatization of amino acids. An additional advantage of this procedure is the high number of biotin molecules that can be attached per Ep molecule. The presence of 8 to 10 biotin molecules per Ep-molecule, as estimated by SDS-PAGE in this study, demonstrates that many of the 9 through 16 sialic acid residues present on each molecule of both urinary as well as BHK-cell-derived recombinant Ep are available for labeling with biotin.

As we were completing this study, Wojchowski and Caslake reported a similar procedure for biotinylating Ep with retention of significant biological activity. These authors used b-Ep in conjunction with 125I-streptavidin to quantitate Ep-receptors on murine erythroleukemia cells but did not present any other applications. The application of b-Ep and 125I-streptavidin to measure dissociation constants and receptor numbers is problematic because it is difficult to determine the number of 125I-streptavidin molecules bound per molecule of receptor-bound b-Ep. Specifically, although each molecule of our b-Ep preparations contains 8 to 10 biotins (Fig 1), it is unlikely that more than a few streptavidin molecules can actually bind to each molecule of receptor-bound b-Ep because of steric hindrance. In addition, the washing step used to remove unbound b-Ep before incubation of cells with 125I-streptavidin most likely leads to dissociation of some b-Ep from the cells, since equilibrium conditions are no longer present. For these reasons binding constants and receptor numbers on hematopoietic cells are more easily determined in single-step binding experiments using radiolabeled Ep.

In this study two novel applications for b-Ep (ie, detection of erythroid cells by flow cytometry and affinity purification of the Ep-receptor) were developed. The labeling of cells with b-Ep and streptavidin-RPE resulted in a sufficiently high fluorescence signal to allow detection of Ep-receptor–positive murine erythroid cells despite the low receptor numbers on these cells. The specificity of the method was demonstrated by the high proportion of b-Ep/streptavidin-positive cells identified in spleens from mice made anemic with phenylhydrazine and the inability to detect positive cells in spleens from untreated mice. This finding is consistent with
previous data showing that more than 90% of the cells in phenylhydrazine-treated mice are Ep-responsive, whereas erythroid cells constitute only a small fraction of total cells in normal spleens, as evidenced by the absence of a significant proliferative response of these cells to Ep (G. Krystal, unpublished observations, January 1983).

Additional evidence for the specificity of the method was provided by the strong positive correlation observed between b-Ep/streptavidin-RPE staining and expression of the transferrin receptor, which is most strongly expressed on cells of the erythroid lineage between the BFU-E and reticulocyte stage. Like the evolution of transferrin-receptor expression during erythroid differentiation, it has been determined by in situ hybridization using 125I-Ep that Ep-receptor expression increases after the BFU-E stage, is maximal at the CFU-E or proerythroblast stage, and declines during terminal differentiation. The analysis of b-Ep/streptavidin-RPE-positive cells in windows for forward- and right-angle light scatter indicated that a relatively large subpopulation of Ep-receptor-positive cells in mouse bone marrow consists of small, late, normoblastlike cells. This indicated that Ep-receptors are present on terminally differentiating erythroid cells. However, since Ep-receptors are undetectable on mature RBCs, it remains to be established at precisely what stage of differentiation, before or after nucleus extrusion, Ep-receptors disappear from the cell surface. The less abundant, larger Ep-receptor-positive bone marrow cells with forward-scatter profiles similar to those of lymphocytes and blast cells most likely represent earlier stages of erythroid differentiation, such as proerythroblasts and CFU-E. Isolation of Ep-receptor-positive cells by FACS should not only prove useful for examining the regulation of Ep-receptor expression during erythroid differentiation but also for studying the mechanism of action of Ep with normal erythroid cells. However, it may be difficult with this approach to examine the functional properties of murine erythroid cells because in preliminary experiments they did not survive the cell-sorting procedure very well (viability less than 10%). Functional analysis of viable erythroid cells sorted on basis of Ep-receptor expression should, however, be possible using, for example, more durable human erythroid cells.

Another potential use of b-Ep is in the purification of the Ep-receptor. Using b-Ep and adsorption to immobilized streptavidin, we have identified the Ep-receptor expressed on COS cells transfected with the murine Ep-receptor cDNA. These cells were used, rather than normal erythroid cells, because they expressed, on average, 100-fold higher receptor numbers than erythroid cells expressing the native receptor. In addition, it allowed metabolic labeling of the receptor using radioactive amino acids, thus facilitating its detection. The receptor, as expressed by the transfected COS cells, consisted of a 65-Kd protein and a less abundant 60-Kd protein. Our results are in agreement with the results of immunoprecipitation experiments using anti-Ep receptor antibodies, which yielded doublet proteins of similar mol wt for the recombinant Ep-receptor. Our results (Figs 7 and 8) also indicate that the 60 Kd protein is localized intracellularly, and preliminary results suggest that the difference between the two proteins may be caused by N-linked glycosylation (A.W. Wognum, unpublished results, October 1989). It remains to be established what the precise relationship is between the 65 Kd Ep-receptor expressed on the cell-surface and the 100- and 85-Kd proteins previously identified by chemical cross-linking of 125I-Ep to the surface of normal and leukemic erythroid cells.

ACKNOWLEDGMENT

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