Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 7574–7578, December 1984
Medical Sciences

Specific binding of erythropoietin to spleen cells infected with the anemia strain of Friend virus

(differentiation/receptors/target cells)

S. B. KRANTZ* AND E. GOLDWASSER†‡

*Division of Hematology, Department of Medicine, Vanderbilt University School of Medicine and Veteran's Administration Medical Center, Nashville, TN 37232; and †Department of Biochemistry and Molecular Biology, The University of Chicago, 920 East 58th Street, Chicago, IL 60637

Communicated by Leon O. Jacobson, July 16, 1984

ABSTRACT **Tritiated erythropoietin with full biological activity has been prepared, and a relatively homogeneous population of enriched progenitor cells that respond to the hormone has been generated by infection of mice with the Friend virus that produces anemia. These cells, obtained from the spleens of infected mice, develop into mature erythroblasts and erythrocytes in the presence of erythropoietin. We have measured the binding of erythropoietin to these target cells; 62% of the binding was inhibited by excess unlabeled erythropoietin, but no inhibition occurred with albumin, serum, or a variety of growth factors and glycoproteins. Apparent equilibrium was reached by 2 hr at 37°C and by 3.5–4 hr at 10°C. The extent of specific binding increased linearly with cell concentration. In binding experiments at 10°C, apparent saturation of specific binding occurred at ≈8.7 nM. Scatchard analysis showed a single class of binding sites. The dissociation constant is 5.2 nM with an average of 660 binding sites per cell. At 0.06 nM, where most of the cells are induced to terminally differentiate *in vitro*, an average of only 8 erythropoietin molecules bound per cell. These studies indicate that erythropoietin attaches to specific binding sites, which are most likely receptors since they manifest high affinity and specificity, and that the biologic effect of the hormone may be produced by attachment of a very small number of erythropoietin molecules.**

Erythropoietin (Epo) is the primary inducer and regulator of red cell differentiation (1). Investigation of the interaction of Epo with erythroid progenitor cells has been hampered by the fact that standard preparations of iodine-labeled Epo are biologically inactive and by a lack of sufficient numbers of pure target cells. Diverse methods of radioiodination cause loss of biological activity (2), presumably because of alterations of a small region of the protein that is necessary for Epo interaction with its target cells. Recently, however, in one of our laboratories (E.G.), Weiss *et al.* (3) introduced ^3H into the oligosaccharide chains of the hormone, through a modification of the sialic acid residues, with retention of >95% of the original biological activity. During the same period, it was shown in the other laboratory (S.B.K.) that infection of murine spleen cells by the anemia strain of the Friend virus (FVA), either *in vitro* (4–6), or *in vivo* (7), leads to a massive proliferation of erythroid progenitor cells that depend upon Epo for terminal erythroid differentiation. These FVA-cells, obtained by plucking clusters ("bursts") of erythroid cells produced in culture (5, 6) or by harvesting infected spleen cells that have been purified by velocity sedimentation at unit gravity (7), represent a large number of relatively homogeneous Epo-responsive target cells. In the present study, we have used tritiated Epo and FVA-cells to characterize specific binding of Epo. Our studies indicate that, through the use of both of these components, it is now possible to measure specific binding of Epo and that the biological effect of the hormone most likely occurs as a result of attachment of a very small number of Epo molecules to specific receptors on the target cells.

MATERIALS AND METHODS

Proteins, Virus, and Cell Lines. Murine epidermal growth factor and nerve growth factor were from Collaborative Research, and human lactoferrin was from Metallo Protein Laboratories (Winston, ON, Canada). Human orosomucoid was prepared from Cohn fraction VI by the method of Whitehead and Sammons (8). FVA was the pseudotype $SFFA_A$/FRE cl-3/MuLV-F(201) obtained from W. D. Hankins at the National Institutes of Health (4) and was maintained by passage of infectious plasma in BALB/c mice. The FDC-P1 cell line, derived from murine marrow cells (9), and the EL-4 cell line, derived from a murine thymoma (10), were gifts from M. B. Prystowsky, Department of Pathology, The University of Chicago.

Purification of Epo. Human urinary Epo Fraction IIIA, (β form) was purified to 70,000 units/mg of protein as described by Miyake *et al.* (11). $NaDodSO_4$/PAGE in the presence of 0.05% Triton X-100 showed only a single symmetrical band for the purified hormone. β Epo (M_r, 34,000) is 24% carbohydrate by weight and its biological activity is ≈53,000 units/mg of Epo (11, 12). In some experiments, partially purified human urinary Epo of 500–1000 units/mg of protein was used to provide an excess of unlabeled Epo; it was the product of the first four steps of the purification procedure.

Preparation of [^{3}H]Epo. Homogeneous Epo (70,000 units/mg of protein) was tritiated in the sialic acid termini by the method of Van Lenten and Ashwell (13) as previously reported (3). Briefly, 40 μl of 0.12 M sodium periodate was added to 100 μg of Epo in 56 μl of 0.1 M sodium acetate/0.15 M sodium chloride, pH 5.6, and the reaction was allowed to proceed for 10 min at 4°C. Five microliters (90 μmol) of ethylene glycol was then added and, after 2 hr at 4°C, the solution was dialyzed overnight at 4°C vs. 0.05 M sodium phosphate/0.15 M sodium chloride, pH 7.4. The oxidized Epo was then reduced with NaB^3H_4 (100 mCi/60 μg, 1 mCi = 37 MBq) for 1 hr at 25°C and dialyzed vs. 0.1 M sodium acetate/0.15 M sodium chloride, pH 5.6, overnight. [^{3}H]Epo was subsequently dialyzed against and then stored in 10% ethylene glycol/10 mM Tris Cl/10 mM $CaCl_2$, pH 7.0, at 4°C. HPLC on a molecular-sizing Toyosoda TSK G3000 SW column (0.75 × 60 cm) was used to separate the single radioactive Epo peak from unreacted NaB^3H_4. Before use in binding studies, the [^{3}H]Epo was dialyzed vs. 0.5% bovine serum albumin/0.15 M NaCl/0.05 M sodium phosphate, pH 7.4, and then concentrated. The final solution contained

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: Epo, erythropoietin; FVA, anemia-producing strain of Friend virus; FVA-cells, FVA-infected hematopoietic cells that are Epo-responsive; SFFV, spleen focus-forming virus.

‡To whom reprint requests should be addressed.

115,000 dpm/μl. The labeled Epo had a specific activity of 7.72 μCi/μg of protein (297,000 dpm/unit, ≈12 ^{3}H/molecule) and retained 95% of its biological activity when assayed by the bone-marrow-culture method (13).

Preparation of FVA-Cells. BALB/c mice were infected by tail vein infusion of 10^4 spleen focus-forming units (14). Sixteen to 21 days later a single cell suspension of spleen cells was made at 4°C as described (7). The cells were suspended in 0.2% deionized bovine serum albumin (15) in Dulbecco's modified Eagle medium (DME medium) and were separated by velocity sedimentation at unit gravity using a modification of the method of Miller and Phillips (16, 17). A 62-ml linear gradient (1.0–2.0%) of deionized bovine serum albumin in DME medium/20 mM 3-(*N*-morpholino)propanesulfonic acid (Mops), pH 7.4, was formed in a 100-ml syringe. The cell suspension (8 ml, 10^8 cells per ml) was layered on the gradient and the cells were allowed to sediment at 4°C for 4 hr; 35 ml was collected from the bottom of the gradient into a 50-ml polypropylene tube and the cells were centrifuged at 200 × *g* for 5 min at 4°C. The cell pellet was suspended in 50% Iscove's modified Dulbecco's medium/50% alpha medium/30 mM $NaHCO_3$/20 mM Mops/200 units of penicillin/ml/250 μg of streptomycin/ml, pH 7.4. In most experiments, the cell medium also contained 15% fetal bovine serum and 1% bovine serum albumin, because FVA-cells are induced to differentiate by Epo in medium that contains these components (5, 6, 18). It has been shown that 95% of these cells are early erythroid cells and that, after a 48-hr incubation with Epo, 86% of the erythroblasts stain with benzidine, indicating the presence of hemoglobin (7).

Binding Method. Microwell cluster plates were preincubated in a 10°C water bath or in an incubator at 37°C with a humidified 5% CO_2 atmosphere. An aliquot (40 μl) of the cell suspension was added to each well, the cells were preincubated for 15 min, and then 20 μl of [^{3}H]Epo, at a known concentration in medium without serum, was added. In experiments requiring centrifugation of the cells, incubations were done in 5-ml polystyrene tubes rather than in microwells. After the desired interval, 0.2 ml of 0.15 M NaCl/0.05 M sodium phosphate, pH 7.4, at 0°C was added to each replicate and the cell suspensions were layered on 0.9 ml of 10% bovine serum albumin/0.15 M NaCl/0.05 M sodium phosphate, pH 7.4, at 0°C, in 1.5-ml Microfuge tubes. The cells were separated from the radioactive medium by centrifugation at 400 × *g* for 5 min at 4°C. (Further centrifugation, at 10,000 × *g* for 3 min, did not increase the radioactivity in the cell pellet, and centrifugation of [^{3}H]Epo alone did not sediment any radioactivity to the bottom of the tube.) The tube contents were frozen using dry ice/ethanol, and the tips were then cut off just above the pellet. The pellet was solubilized in 0.5 ml of 0.1% $NaDodSO_4$ and the radioactivity was measured by liquid scintillation counting, using counting times sufficient to ensure precision within 3%.

Nonspecific binding was measured by the same method, except that a 200-fold excess of unlabeled Epo was added with the [^{3}H]Epo. Specific binding is the difference between total and nonspecific binding. All experiments were performed in duplicate or triplicate. The average range of the experimental values from the mean was 8.5% of the mean. Standard errors of the mean, or standard errors in the differences of the means, were calculated for all means with more than two replicates, and significance was calculated using the Student *t* test (19).

RESULTS

Time Course of Binding. Times courses of specific [^{3}H]-Epo binding to FVA-cells are shown in Fig. 1. At 37°C, maximum binding was achieved within 2 hr and remained stable for an additional 2 hr. At 10°C, the rate of binding was significantly decreased and plateaued at 3.5 hr. At this temperature, internalization is greatly reduced (20), but maximum binding was nearly that observed at 37°C. Since binding at 3.5 hr was not significantly different from that at 4 hr ($0.4 < P < 0.5$), binding was measured at 3.5 hr in all subsequent experiments. At 24°C, the rate of binding was only slightly decreased from that at 37°C, while at 4°C the rate was much less (data not shown).

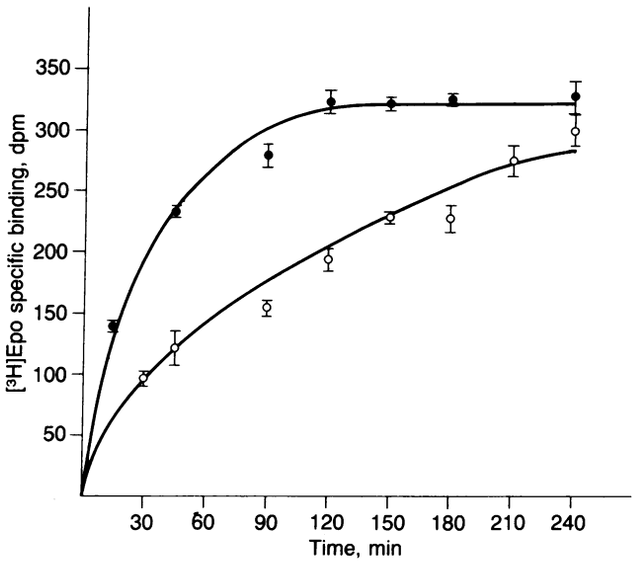

FIG. 1. Time course of erythropoietin binding to FVA-cells. [^{3}H]Epo (380 pM) was incubated with 8.7 × 10^6 FVA-cells in 60 μl of standard medium at 37°C (●) or 10°C (○). At the indicated times, cell-associated [^{3}H]Epo was measured and corrected for nonspecific binding. Each point is the mean of triplicates.

Specificity of Binding. In one experiment, 240- and 480-fold excesses of unlabeled Epo reduced total [^{3}H]Epo binding to FVA-cells by 57% (Table 1). In 14 experiments with a 200-fold excess of unlabeled Epo, specific binding was 62 ± 2.9% of total binding. The quantitative aspects of this competition are shown in Table 2. Neither any of a variety of growth factors, nor bovine serum albumin, nor fetal bovine serum competed with [^{3}H]Epo for binding to FVA-cells, even though they were present in great excess (Table 1). Orosomucoid, a glycoprotein similar to Epo in molecular weight and sugar composition, also did not compete with [^{3}H]Epo. To determine whether the lack of complete inhibition of binding of [^{3}H]Epo by unlabeled Epo was due to al-

Table 1. Specificity of [^{3}H]Epo binding to FVA-cells

Addition	Total binding, dpm
None	420 ± 9
Erythropoietin, 70 nM	180 ± 7
Erythropoietin, 140 nM	180 ± 4
Albumin, 1%	360 ± 18
Fetal bovine serum, 10%	380 ± 4
Albumin, 1% + fetal bovine serum, 10%	380 ± 25
Orosomucoid, 140 nM	360 ± 57
Nerve growth factor, 350 nM	460 ± 9
Epidermal growth factor, 770 nM	400 ± 10
Insulin, 140 nM	440 ± 9
Lactoferrin, 140 nM	430 ± 18

[^{3}H]Epo (290 pM) was incubated at 10°C with 9.6 × 10^6 FVA-cells in 60 μl of medium (50% alpha medium/50% Iscove's modified Dulbecco's medium) with the above additions. After 3.5 hr the amount of [^{3}H]Epo bound to the FVA-cells was determined. Each value is the mean ± SEM ($n = 3$). The difference between 420 dpm and 180 dpm is significant ($P < 0.001$).

Table 2. Competition for binding sites by unlabeled Epo

Unlabeled Epo, nM	Total binding, dpm	Specific binding, % of total
0	188 ± 4.5	0
0.03	203 ± 4.2	0
0.11	185 ± 4.2	1.6
0.22	179 ± 4.0	4.8
0.44	121*	35.6
1.1	121 ± 3.8	35.6
2.2	121 ± 2.8	35.6
3.3	108 ± 1.5	42.6
4.4	104*	44.7
11.1	84.3 ± 2.0	55.2
18.3	78.7 ± 2.4	58.1
183	61.3 ± 1.8	67.4

[^{3}H]Epo (0.31 nM) was incubated at 10°C with 4.5 × 10^6 FVA-cells in 60 μl of standard medium containing various concentrations of unlabeled Epo for 3.5 hr prior to measurement of total [^{3}H]Epo binding, Each total binding is the mean ± SEM (n = 3), unless otherwise indicated.
*Mean of duplicates.

teration of the radioactive hormone by the tritiation procedure, which removes 2 carbons, a nonradioactive Epo was prepared using the procedure for tritiation, but with unlabeled $NaBH_4$. This product retained full biological activity and, at a 500-fold excess concentration, it competed with [^{3}H]Epo binding to FVA-cells to the same degree as pure Epo (data not shown). [^{3}H]Epo that was accidentally prepared in the presence of excess sodium hydroxide, which was biologically inactive, did not show any specific binding to FVA-cells. No specific binding of [^{3}H]Epo was observed with normal murine thymocytes, splenocytes, or a variety of cell lines (Table 3).

Effect of Cell Concentration and [^{3}H]Epo Concentration on Binding. When the FVA-cell concentration was increased, there was a proportional increase in the amount of [^{3}H]Epo specifically bound to the cells after 3.5 hr at 10°C (Fig. 2), indicating that the Epo concentration was not limiting. The effect of [^{3}H]Epo concentration on specific binding is shown in Fig. 3. Apparent saturation of specific Epo binding occurred at a concentration of ≈8.7 nM. A Scatchard plot of these data (shown in the inset of Fig. 3) indicates that a single class of Epo receptors on the FVA-cells is detected at the concentrations of Epo used. In three separate experiments, the mean K_d was 5.2 nM (range, 4.4–6.2 nM). An average of ≈660 binding sites were present per FVA-cell, as determined from the Scatchard plots.

Characteristics of [^{3}H]Epo Binding. To determine whether bound Epo dissociates from FVA-cells, the cells were first incubated with [^{3}H]Epo at 10°C and were then centrifuged through 10% bovine serum albumin to remove unbound Epo. The cells were resuspended in the medium used for the binding experiments and were incubated under the binding conditions for an additional 3.5 hr. The [^{3}H]Epo remaining bound was then measured. Of the 390 dpm originally bound to the cells, ≈40% (160 dpm) remained after 3.5 hr incubation at 10°C (Table 4). In the presence of 630 nM unlabeled Epo, the results were similar. Gel permeation HPLC of the released radioactive material showed a single radioactive species, which had the same apparent molecular size as the original [^{3}H]Epo (data not shown). At 37°C, the FVA-cells bound a greater amount of [^{3}H]Epo and, when subsequently incubat-

Table 3. Binding of [^{3}H]Epo to different cells

Cells	[^{3}H]Epo binding, dpm		
	Total	Nonspecific*	Specific
Normal mouse thymocytes	35 ± 1	46 ± 25	0
Normal mouse splenocytes	71 ± 36	92 ± 43	0
FDC-P1	80 ± 45	77 ± 29	3
EL-4	85 ± 52	118 ± 44	0
FVA-cells	215 ± 9	52 ± 7	163

[^{3}H]Epo (230 pM) was incubated at 10°C with 5 × 10^6 cells (except for EL-4: 1.4 × 10^6) in standard medium for 3.5 hr before measurement of [^{3}H]Epo binding. Each value is the mean ± SEM (n = 3). The difference between total and nonspecific binding for FVA-cells is significant ($P < 0.001$).
*In the presence of 90 nM unlabeled Epo.

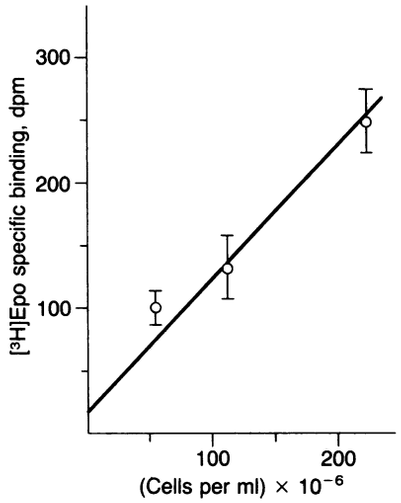

FIG. 2. Effect of cell concentration on erythropoietin binding to FVA-cells. Various concentrations of FVA-cells were incubated with [^{3}H]Epo (560 pM) in 60 μl of medium at 10°C for 3.5 hr. Cell-associated [^{3}H]Epo was measured and corrected for nonspecific binding. Each point is the mean of triplicates.

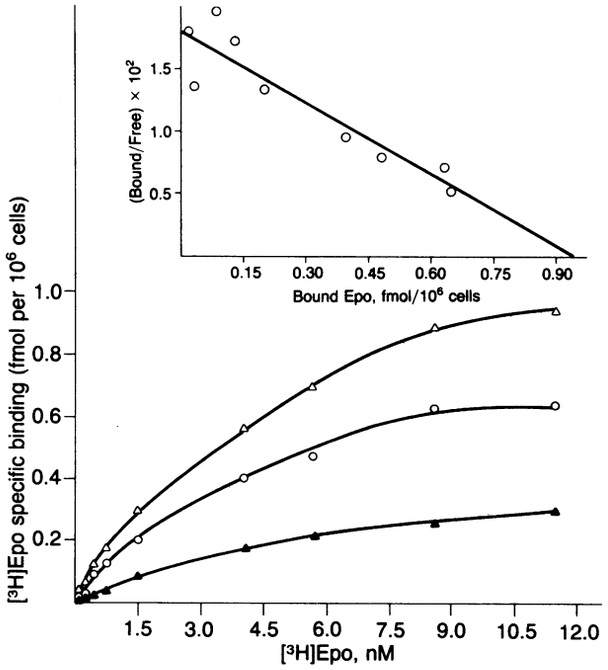

FIG. 3. Effect of erythropoietin concentration on binding to FVA-cells. Various concentrations of [^{3}H]Epo were incubated with 5.6 × 10^6 FVA-cells in 60 μl of medium at 10°C for 3.5 hr (△). Replicates had a 200-fold excess of unlabeled Epo (▲). Cell associated [^{3}H]Epo was then measured and specific binding (○) was calculated by subtracting the nonspecific binding (▲) from the total binding (△). Each point is the mean of duplicates. *Inset*: Scatchard plot by linear regression analysis of the data.

Table 4. Dissociation of [^{3}H]Epo and stability of [^{3}H]Epo or receptor

Sample	Incubation		[^{3}H]Epo bound,* dpm
	First	Second	
1	[^{3}H]Epo	No incubation	390 ± 16
2	[^{3}H]Epo	No addition	160 ± 7
3	[^{3}H]Epo	Unlabeled Epo	130 ± 13
4	[^{3}H]Epo	No incubation	540 ± 20
5	[^{3}H]Epo	No addition	270 ± 17
6	[^{3}H]Epo	Unlabeled Epo	240 ± 1
7	[^{3}H]Epo	No incubation	280 ± 7
8	No addition	s7	240 ± 5
9	No addition	[^{3}H]Epo	360 ± 58

All incubations were at 10°C, except the first incubation for samples 4–6, which was at 37°C. Triplicate samples 1–9 were processed as follows. First incubation: 6.1×10^6 FVA-cells were incubated in 5-ml polystyrene tubes with standard medium with or without 360 pM [^{3}H]Epo. Second incubation: Samples 1–6 were layered on 2 ml of 10% bovine serum albumin/0.15 M NaCl/0.05 M sodium phosphate, pH 7.4, and centrifuged at $400 \times g$ for 5 min at 4°C. Binding in samples 1 and 4 was determined without further incubation. Cells from samples 2, 3, 5, and 6 were incubated for 3.5 hr in fresh medium with or without 630 nM unlabeled Epo before binding was measured. Samples 7–9 were centrifuged at $200 \times g$ for 5 min at 4°C. Sample 7 was not incubated further. Sample 8 was incubated with the sample 7 supernatant (s7) for 3.5 hr. Sample 9 was incubated for the same time in medium containing 360 pM [^{3}H]Epo.

*Each value is the mean ± SEM ($n = 3$). The differences between samples 1 and 2 and between samples 4 and 5 are significant ($P < 0.001$).

ed at 10°C, also released a greater amount, which was only slightly increased when 630 nM Epo was present in the medium.

The stability with respect to binding of [^{3}H]Epo under the above conditions was determined by removing the [^{3}H]Epo-containing supernatant from FVA-cells after a 3.5 hr incubation at 10°C and incubating it with FVA-cells that had not been exposed to Epo. The [^{3}H]Epo in the supernatant bound to FVA-cells as well as did fresh ^{3}H-Epo (see Table 4, samples 8 and 7, respectively). To test the stability of the cells' capacity to bind [^{3}H]Epo, FVA-cells were incubated in the binding medium without [^{3}H]Epo for 3.5 hrs at 10°C. The medium was then removed and fresh medium containing [^{3}H]Epo was added to these cells. Binding under these conditions was essentially the same as that found for fresh FVA-cells (Table 4, samples 9 and 7). No significant binding of [^{3}H]Epo to the plastic wells was apparent. Thus, [^{3}H]Epo and its cellular binding sites are quite stable at 10°C in the medium used. Dissociation of the Epo-receptor complex in the absence of exogenous Epo suggests that the binding is reversible.

DISCUSSION

In bone marrow and spleen, 0.01–0.5% of the cells are erythroid progenitor cells (21, 22). Based on the time of appearance, the size, and the configuration of erythroid colonies they produce in cell cultures, the erythroid progenitor cells have been classified into three principal categories: the erythroid colony-forming units, the mature erythroid burst-forming units, and the immature erythroid burst-forming units, which may require another growth factor (21–24). The expansive development of erythroid progenitor cells in response to Epo eventually leads to terminal differentiation into late erythroblasts that do not require Epo for further development and that, along with other hematopoietic cells, greatly outnumber the responsive cells (25).

Weiss *et al.* (26) used both an indirect immunofluorescence method and a direct fluorescence technique to find that ≈1.3% of the cells in normal marrow are capable of binding Epo. In addition, they showed that [^{3}H]Epo bound specifically to a population of mouse spleen cells enriched in erythroid colony-forming units and to normal rat marrow cells (13). Friend retrovirus complexes, consisting of replication-defective spleen focus-forming virus (SFFV) and replication-competent murine leukemia virus (27) infect erythroid progenitor cells and rapidly cause an increase in their number. The SFFV is competent for erythroid transformation *in vitro* and acts on the mature erythroid burst-forming unit, its principal target cell, to initiate development into erythroid bursts in 5 days without the addition of Epo (28). The cells of the erythroid bursts produced in response to SFFV of FVA ($SFFV_A$) remain without hemoglobin and other proteins of terminal differentiation for several days (4–7). These cells, when subcultured with Epo, give rise to 2- to 32-cell colonies of erythroblasts in two days. The $SFFV_A$-induced erythroid bursts, which can be plucked from the cultures, consist of $2–3 \times 10^3$ Epo-responsive cells per burst (6); these constitute a relatively homogeneous group of cells sensitive to the hormone, but they are not available in large quantity. Recently, however, Koury *et al.* (7) have shown that the spleen cells of mice infected two weeks earlier with FVA are almost entirely replaced by Epo-responsive cells equivalent to those produced *in vitro*. These observations have made possible the study of the interaction of Epo with its target cells.

In this paper, we have described specific binding of Epo to FVA-cells, with an average of 660 binding sites per cell. Previous evidence for Epo action on the cell membrane has consisted of (*i*) reversible obliteration of Epo responsiveness by trypsin treatment of marrow cells (29), (*ii*) the biological activity *in vitro* of Epo attached to agarose beads (30), and (*iii*) the attachment of a fluorescent adduct of Epo to a small fraction of normal rat marrow cells (26). The present work provides additional direct evidence that Epo attaches to cell-surface binding sites, which are most likely receptors since they manifest high binding affinity ($K_d = 5.2$ nM) and specificity. Burgess (31) has reported that normal myeloid target cells, which are induced into granulocyte/macrophage differentiation by colony-stimulating factor, also have a low concentration of receptors for their inducer; it is possible that a low receptor number may be charactristic of many of the cells responsive to factors that induce hematopoietic differentiation. Mononuclear phagocytes, however, have 3,000–16,000 receptors for colony-stimulating factor 1 per cell (32).

Because of the heterogeneity of erythroid colonies arising from FVA-cells, the number of receptors per cell may vary widely. In addition, FVA-infected cells may have a different number of Epo binding sites than do normal erythroid progenitors; the latter may be even more heterogeneous with respect to Epo binding sites, since they range from the earliest erythroid burst-forming unit, with low Epo sensitivity, to a cell that is very close temporally to an erythroblast and has very high sensitivity to Epo (22, 33). Further studies will be needed to measure the range of Epo binding sites on normal erythroid progenitors before any conclusions can be made on the number of Epo binding sites of these cells. This will be quite difficult, not only because of the low concentration of normal Epo-responsive cells, but also because the cells are commonly generated by increasing blood Epo levels (34), which may alter the number of Epo binding sites.

Epo stimulates hemoglobin synthesis in FVA-cells to the same extent and with a similar time course as it does in normal erythroid colony-forming units (7, 21). Since most of the cells respond to Epo by synthesizing hemoglobin, a large variation in the number of specific binding sites per cell would allow the same conclusion as the small average num-

ber of binding sites: the biologic effect of Epo is most likely produced by the binding of a very small number of Epo molecules. At a concentration of 0.06 nM, where most of the FVA-cells are induced to terminally differentiate, an average of only 8 molecules are bound per cell, based on our measurements of the relation of Epo concentration to Epo binding (Fig. 3). This explains the puzzling observation (35) that, after marrow cell incubation with Epo and marked stimulation of erythropoiesis *in vitro*, Epo levels in the medium remained relatively unchanged.

The characteristics of the specific binding of Epo to the FVA-cells at 10°C indicate that this is most likely an equilibrium reaction, with the hormone remaining intact and active on the cell membrane, as well as in the medium, and freely dissociating. It is possible that some of the Epo may be internalized at 37°C. However, since only a few Epo molecules are probably enough to produce the biological effect of the hormone, the methods used here are not sensitive enough to detect internalization of so little Epo and do not allow us to draw any conclusions about the relation of internalization to the effect on hemoglobin synthesis. Isolating the specific Epo receptor and determining its structure and function should allow insight into any second-messenger mechanism by which Epo stimulates nuclear RNA transcription shortly after its application to target cells. The present studies, which identify specific binding of Epo to its target cells, now make possible isolation and characterization of the Epo receptor and production of antibodies to it.

The authors wish to thank Drs. Mark J. Koury, Steve T. Sawyer, and Maurice C. Bondurant for sharing their FVA-cell system prior to its publication and for all their advice and help. This work was supported in part by the Veterans Administration, by National Institutes of Health Grants HL 21676, CA 18375, and AM 15555, by the Blossom and Irving Levin Fund for Cancer Research and by the Vanderbilt University Research Council.

1. Krantz, S. B. & Jacobson, L. O. (1970) *Erythropoietin and the Regulation of Erythropoiesis* (The University of Chicago Press, Chicago).
2. Goldwasser, E. (1981) in *Control of Cellular Division and Development*: Part A, eds. Cunningham, D., Goldwasser, E., Watson, J. & Fox, C. F. (Liss, New York), pp. 487–494.
3. Weiss, T. L., Kung, C. K.-H. & Goldwasser, E. (1983) in *Normal and Neoplastic Hematopoiesis*, eds. Golde, D. W. & Marks, P. A. (Liss, New York), pp. 455–463.
4. Hankins, W. D. & Troxler, D. (1980) *Cell* **22,** 693–699.
5. Koury, M. J., Bondurant, M. C., Duncan, D. T., Krantz, S. B. & Hankins, W. D. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 635–639.
6. Bondurant, M., Koury, M., Krantz, S. B., Blevins, T. & Duncan, D. T. (1983) *Blood* **61,** 751–758.
7. Koury, M. J., Sawyer, S. T. & Bondurant, M. C. (1984) *J. Cell Physiol.*, in press.
8. Whitehead, P. H. & Sammons, H. G. (1966) *Biochim. Biophys. Acta* **124,** 209–211.
9. Ihle, J. N., Keller, J., Greenberger, J. S., Henderson, L., Yetter, R. A. & Morse, H. C., III (1982) *J. Immunol.* **129,** 1377–1383.
10. Hilfiker, M. L., Moore, R. N. & Farrar, J. J. (1981) *J. Immunol.* **127,** 1983–1987.
11. Miyake, T., Kung, C. K.-H. & Goldwasser, E. (1977) *J. Biol. Chem.* **252,** 5558–5564.
12. Dordal, M. (1982) Dissertation (The University of Chicago, Chicago).
13. Van Lenten, L. & Ashwell, G. (1971) *J. Biol. Chem.* **246,** 1889–1894.
14. Axelrad, A. A. & Steeves, R. A. (1964) *Virology* **24,** 513–515.
15. Worton, R. G., McCulloch, E. A. & Till, J. E. (1969) *J. Cell. Physiol.* **74,** 171–182.
16. Miller, R. G. & Phillips, R. A. (1969) *J. Cell. Physiol.* **73,** 191–202.
17. Koury, M. J. & Krantz, S. B. (1982) *Cell Tissue Kinet.* **15,** 59–67.
18. Kost, T. A., Hankins, W. D. & Krantz, S. B. (1980) *Exp. Hematol. Suppl. 8,* **8,** 248–258.
19. Snedecor, G. W. & Cochran, W. G. (1967) *Statistical Methods* (Iowa State Univ. Press, Ames, IA), 6th Ed.
20. Steinman, R. M., Mellman, I. S., Muller, W. A. & Cohn, Z. A. (1983) *J. Cell Biol.* **96,** 1–27.
21. McLeod, D. L., Shreeve, M. M. & Axelrad, A. A. (1974) *Blood* **44,** 517–534.
22. Axelrad, A. A., McLeod, D. L., Shreeve, M. M. & Heath, D. S. (1974) in *Proceedings of the Second International Workshop on Hemopoiesis in Culture*, ed. Robinson, W. A. (DHEW Publication NIH 74-205, Washington, DC), pp. 226–237.
23. Gregory, C. J. (1976) *J. Cell. Physiol.* **89,** 289–301.
24. Iscove, N. N. (1977) *Cell Tissue Kinet.* **10,** 323–334.
25. Filmanowicz, E. & Gurney, C. W. (1961) *J. Lab. Clin. Med.* **57,** 65–72.
26. Weiss, T. L., Kung, C. K.-H. & Goldwasser, E. (1984) *J. Cell. Biochem.*, in press.
27. Troxler, D. H., Ruscetti, S. K. & Scolnick, E. M. (1980) *Biochim. Biophys. Acta* **605,** 305–324.
28. Kost, T. A., Koury, M. J. & Krantz, S. B. (1981) *Virology* **108,** 309–317.
29. Chang, S. C.-S., Sikkema, D. & Goldwasser, E. (1974) *Biochem. Biophys. Res. Commun.* **57,** 399–405.
30. Roodman, G. D., Spivak, J. L. & Zanjani, E. D. (1981) *J. Lab. Clin. Med.* **98,** 684–690.
31. Burgess, A. W. (1984) *Lymphokine Res.* **3,** 64 (abstr.).
32. Stanley, E. R., Guilbert, L. J., Tushinski, R. J. & Bartelmez, S. H. (1983) *J. Cell. Biochem.* **21,** 151–159.
33. Ouellette, P. L. & Monette, F. C. (1980) *J. Cell. Physiol.* **105,** 181–184.
34. Nijhof, W. & Wierenga, P. K. (1983) *J. Cell Biol.* **96,** 386–392.
35. Krantz, S. B. & Goldwasser, E. (1965) *Biochim. Biophys. Acta* **108,** 455–462.