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The Frequency of Bone Marrow Cells That Bind Erythropoietin

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With a statistical addendum by Paul Meier

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The frequencies of rat and mouse bone marrow cells capable of binding erythropoietin were studied by both direct fluorescence and indirect immunofluorescence. We found that between 1–2% of the cells bound erythropoietin, that the binding was specific, and that the number of cells that bound erythropoietin was, in part, a function of the erythropoietic state of the donor animal. A statistical method for evaluating the data obtained is included.

Key words: erythropoietin, bone marrow, immunofluorescence, differentiation

The glycoprotein erythropoietin (epo) acts on hemopoietic cells, devoid of overt erythroid characteristics, to both induce and maintain normal red cell differentiation [1]. There has been no convincing evidence demonstrating the binding of epo to cells. Indirect evidence, however, suggests that it requires one or more proteins on the external cell surface in order for epo to have an effect on transcription [2]. We show here, by two different methods, that epo binds to cells. Indirect immunofluorescence microscopy, using a monoclonal antibody directed against epo [3], showed that 1.3% of normal mouse marrow cells bind epo after 1 hr at 37°C. Using direct fluorescence microscopy with a fluorescent adduct of epo, we found that 1.5% of normal rat marrow cells bind the hormone. The epo-cell interaction was specific, and the frequency of rat cells capable of binding epo depends on the extent of erythropoiesis.

MATERIALS AND METHODS

To study its properties, the fluorescent-epo adduct was prepared by incubating 0.24 nmoles (8 μg) of pure epo [4] with 20 nmoles of N-7-dimethylamino-methyl coumarinyl maleimide [5] (DACM, Polysciences, Inc., Warrington, PA) in a final volume of 200 μl of 40 mM phosphate buffer at pH 7.25 for 2 hr at 25°C and then for an additional 22 hr at 4°C.

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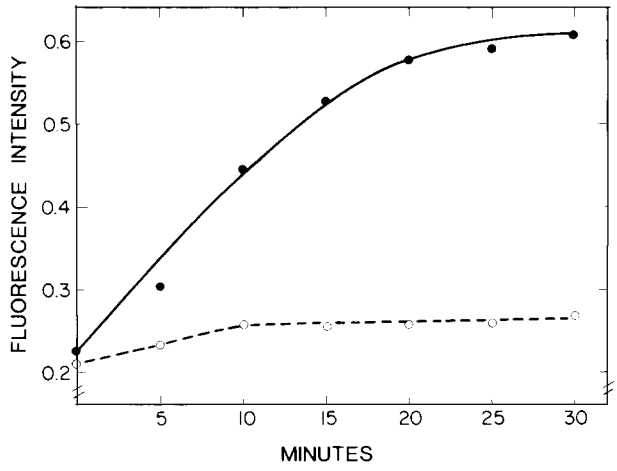

Fig. 1. Time course of the reaction of DACM with erythropoietin. The relative fluorescence was measured for epo incubated with DACM (●—●) and DACM alone (○—○) over a 30-min period.

The time course of the reaction was followed by measuring the increase in fluorescence with an Aminco-Bowman spectrofluorimeter using an excitation wavelength of 400 nm and an emission wavelength of 485 nm. Figure 1 shows the changes in fluorescence over the first 30 min of the reaction. In the presence of epo, we found the increase in fluorescence expected for the reaction of DACM with a thiol, whereas with DACM alone there was no appreciable change in fluorescence. This study also showed that fatty acid-free bovine serum albumin (BSA) reacted with DACM to generate fluorescence, whereas performic acid oxidized BSA, a thiol-free control, did not.

After the 22-hr incubation, the reaction mixture was dialyzed against 1% sodium dodecyl sulfate (SDS) 5 mM phosphate, 20 mM sodium acetate, 2 mM EDTA, pH 7.4, with a continuous flow of 7.5 ml per hr for 22 hr. We determined that DACM was bound to epo by electrophoresing the putative adduct under denaturing and reducing conditions. At the same time, a second gel was run with ^{125}I-epo [4] and molecular weight markers. The second gel was stained with Coomassie blue to determine the marker positions, and both gels were sliced into 2 mm sections. The slices were shaken overnight in 0.2 ml of 1% SDS, 0.15 M NaCl, pH 7.0, at 25°C, and the fluorescence of the supernatant solutions was measured. Radioactivity of the slices of the other gel was measured. We found a fluorescent peak coincident with the ^{125}I-epo at a position in the gel equivalent to an apparent molecular weight of 34,000 (Fig. 2). There were, in addition, smaller fluorescent peaks at integral multiples of this molecular weight, probably representing aggregates of epo and what appears to be a very large aggregate at the top of the gel. There were also some fluorescent peaks smaller than epo, which may represent degradation products formed during the reaction. The strong fluorescent signal near the bottom of the gel is due to the tracking dye pyronin Y.

The DACM-epo used for study of binding to cells was prepared by the original method of Yamamoto et al [5] but the present study included a carrier protein and labeled epo to facilitate determination of recovery. The following were added to 250 μl of 40 mM phosphate buffer: pH 7.25:0.51, nmoles (17.5 μg) of epo, 12,000

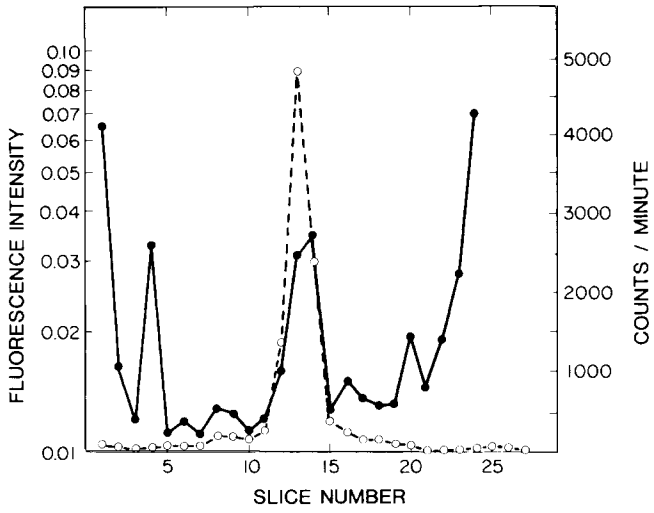

Fig. 2. Electrophoretic analysis of DACM-erythropoietin and ^{125}I-erythropoietin. Fluorescence is indicated by (●—●) and ^{125}I by (○—○). Slice no. 1 is at the top of the gel.

cpm of ^{125}I-epo, 100 μg of performic acid oxidized BSA (used as a thiol-free carrier protein), and 3.9 nmoles of DACM in 25 μl of acetone. The reaction was carried out for 60 min in an ice bath. A 50 μl aliquot of 14.3 mM β-mercaptoethanol in 0.1M phosphate buffer, pH 7.0, was then added to react with excess DACM for an additional 100 min at 0°C. The mixture was dialyzed for 22 hr against a continuous flow of 5 mM phosphate buffer, pH 7.2, at a flow rate of 7.5 ml/hr. The 300 μl sample was diluted with 1.7 ml of 0.1% BSA, 0.15 M NaCl, 0.01 M $CaCl_2$, pH 7.4. The total radioactivity recovered was 85%. Using an assay for epo which measures hemoglobin synthesis in marrow cell cultures [6] we found that approximately 85% of the original biological activity was recovered.

Some recent unpublished data from this laboratory [Wang FF, Kung CKH, Goldwasser E] indicate that there are no free sulfhydryl groups in native epo. The reaction of DACM with epo to generate a fluorescent adduct may involve another accessible residue, possibly histidine.

The monoclonal antiepo:biotin complex was prepared [7] by incubating 60 μg of biotin succinimide in dimethyl sulfoxide with 1 mg of monoclonal antiepo IgG in 1 ml of PBS at 25°C for 4 hr. The unconjugated biotin was removed by overnight dialysis against PBS at 4°C.

Normal mouse marrow cells from the femurs and tibias of 6-week-old female BDF_1 mice were suspended in alpha medium (Gibco, Grand Island, NY), supplemented with 15% fetal calf serum (Flow Laboratories, Rockville, MD), and 50 μg/ml of gentamycin (Shering Corp., Kenilworth, NJ). Three 1 ml samples were prepared at a cell concentration of 50×10^6 cells/ml. One experimental sample with 1.1 units of epo/ml and two control samples with an equal volume of diluent—0.1% bovine serum albumin (BSA), 0.15M NaCl, 10 mM $CaCl_2$—were incubated at 37°C for 1 hr in a 5% CO_2:95% air atmosphere and then layered over 1 ml of 10% BSA in 10 mM phosphate, 0.14 M NaCl, pH 7.2 (PBS) and centrifuged at 100 × g for 10 min at 4°C. The cell pellets from the experimental group and from one control were resuspended in 50 μl of medium containing 0.1 mg/ml of the monoclonal antiepo

biotin complex. The other control was resuspended in 50 μl of medium. The cell suspensions were kept at 4°C for 15 min and then centrifuged through 10% BSA cushion. All three cell pellets were then resuspended in PBS containing saturating levels of an avidin-rhodamine complex (Vector Laboratories, Inc. Burlingame, CA) and kept on ice for 15 min. The cells were again centrifuged through a 10% BSA cushion, resuspended in 10 μl of horse serum, and prepared for fluorescence microscopy by allowing a smear to air dry on a microscope slide prior to the addition of 90% glycerol, 10% PBS, and a coverslip. The total number of cells and the number of fluorescent cells were determined from photographs taken using phase contrast and epifluorescence microscopy of each field. From 40 to 70 fields per sample, from a minimum of two separate experiments, were examined. The total of number of cells counted per sample ranged between 3000–7000.

Rats were made hypoxic by keeping them at 0.5 atmosphere for 20 hr prior to use as a source of marrow. They were made plethoric with respect to red cells by keeping them at 0.5 atmosphere for three weeks then at 1 atmosphere for one week prior to killing. Hemolytic anemia was induced in rats by two intraperitoneal injections of 50 mg/kg of neutralized phenylhydrazine-HCl on two successive days; the rats were killed for marrow cell collection 48 hr later.

Rat marrow cell suspensions were prepared for direct fluorescence studies, as described, and incubated with 1.1 units of DACM-epo/ml of culture for 1 hr at 37°C. The control group was incubated with 0.1% BSA in 0.15M NaCl, 0.01 M $CaCl_2$, or with underivatized DACM. A 30 μl aliquot of propidium iodide (50 μg/ml in PBS) (Sigma Chemical, St. Louis, MO) was added to 1 ml of culture, and cells were centrifuged through a cushion of 1 ml of 10% BSA in PBS at 100 × g for 10 min at 4°C and prepared for microscopy as described above. For each data point we examined 24–60 fields and photographed 1000 to 6000 cells for statistical evaluation. The dead cells which were counterstained with propidium iodide were not included in the analysis. The frequency of fluorescent cells was determined, and the significance of the observed differences was calculated using the statistical analysis described in the addendum to this paper. The number of CFU-e [8] was determined by the two-day colony assay of Iscove and Sieber [9].

The Friend cells used were line GM-86, clone 745, kindly supplied by Dr. David Hankins (The National Cancer Institute), and the K562 cells were a gift from Dr. Michael Horton (St. Bartholomew's Hospital, London).

RESULTS AND DISCUSSION

We found by using monoclonal anti-epo in indirect immunofluorescence studies that erythropoietin is bound to mouse marrow cells and that $1.3 \pm 0.4\%$ ($P < 0.05$) of the total nucleated cells have sufficient receptors to be detectable by this method (Table I). The two control marrow samples incubated with the diluent contained 0.7% or less of fluorescent cells. Because propidium iodide, a fluorescent nuclear stain specific for dead cells, is not distinguishable from rhodamine, we could not determine the percentage of dead cells in these marrow samples. We found, however, that dead cells in similar marrow cultures without rhodamine represent approximately 0.4% of the total, and assume that the frequency of dead cells is the same in control as in the experimental samples. The net percentage of mouse marrow cells that bind epo is

TABLE I. Estimation of Erythropoietin-binding Cells by Indirect Immunofluorescence

Erythropoietin	Antibody:biotin	Avidin-Rhodamine	Percent fluorescent cells
–	–	+	0.7 ± 0.2%
–	+	+	0.6 ± 0.1%
+	+	+	2.0 ± 2.2%
	Experimental-Control		1.3 ± 0.4%($P<0.05$)

Normal mouse marrow cells were incubated with erythropoietin or its diluent followed by monoclonal anti-erythropoietin labeled with biotin and then with rhodamine-labeled avidin.

about twofold greater than the percent of CFU-e (0.55%) determined for the same population of cells.

Use of direct fluorescence to compare the frequency of epo-binding cells in normal rat marrow incubated with DACM-epo, with DACM only, or with the diluent showed that 1.5±0.5% ($P < 0.001$) of the cells specifically bound DACM-epo (Table II). This binding was not observed at 0°C, and the number of fluorescent cells was reduced to the baseline level when a 100-fold excess of unmodified epo was used to compete with the DACM-epo for binding sites. Red blood cells and thymocytes showed no fluorescent cells in any field examined. Neither Friend erythroleukemic cells nor K562 cells showed any significant increase in fluorescence frequency when incubated with DACM-epo. For both of these cell lines, the baseline (no DACM-epo) was considerably higher than for marrow cells, and a small increment might have been undetected.

We conclude that the observed cellular binding of DACM-epo is specific and does not occur with at least two nontarget cells. In determining the physiological relevance of cellular binding of DACM-epo, we found that the fraction of cells that can bind epo reflects the erythropoietic state of the rat marrow (Table II). As expected, hyperplastic marrow from the anemic animals had a higher frequency of epo-binding cells, and the cells from plethoric animals had a lower frequency than that found in the normal cells. The finding of fewer epo-binding cells in the population from rats exposed to hypoxia for 20 hr was unexpected and we tentatively ascribe that result to a greater occupancy of binding sites on the cells by endogenous, nonfluorescent epo formed by the rats in response to acute hypoxia. The marrow cells from the anemic rats may have lower occupancy because of the longer time these rats were anemic and consequent lower epo concentration at the time the cells were taken. These findings have to be corroborated, and further experimentation is needed to help explain them.

These results indicate, by indirect as well as direct fluorescence microscopy, that 1.3–1.5% of normal mouse and rat marrow cells bind erythropoietin. This value is significantly greater than the frequency of CFU-e found for the same mouse cell population, suggesting that cells not capable of forming colonies (probably later than CFU-e in the differentiating pathway) still possess enough binding sites to make them fluoresce.

In earlier investigations, epo antibody interactions were used to study both epo producing and responsive cells [10,11,12] but, because the immune serum was

TABLE II. Estimation of Erythropoietin-Binding Cells by Direct Fluorescence*

Marrow	Conditions	Control	DACM-Epo	Difference	Significance
Normal	37°	0.8 ± 0.2%	2.3 ± 0.3%	1.5 ± 0.4%	$P<0.001$
Normal[a]	37°	0.8 ± 0.2%	—	—	—
Normal	0°	1.4 ± 0.4%	0.9 ± 0.4%	0.5 ± 0.6%	NS[c]
Normal[b]	37°	0.9 ± 0.2%	1.0 ± 0.2%	0.1 ± 0.3%	NS[c]
Phenylhydrazine treated	37°	1.9 ± 0.3%	6.4 ± 0.6%	4.5 ± 0.7%	$P<0.001$
Hypoxic	37°	0.8 ± 0.3%	0.8 ± 0.3%	0.0 ± 0.4%	—
Plethoric	37°	1.0 ± 0.2%	1.8 ± 0.3%	0.8 ± 0.4%	$P<0.05$
Red cells	37°	0.0%	0.0%	—	—
Thymocytes	37°	0.0%	0.0%	—	—

*Rat marrow cells were incubated with DACM-erythropoietin and fluorescent positive cells scored directly. The controls were incubated with no erythropoietin. All cultures were incubated for 1 hr at 37°C except for the sample at 0°C.

[a]Incubated with DACM in the absence of epo.

[b]Incubated with 1.1 units of DACM-epo and 120 units of unlabeled epo.

[c]Not significant.

prepared with very impure epo as the immunogen and has not been shown to be monospecific, these studies cannot be interpreted as demonstrating unequivocally that epo was bound specifically by or present in the cells. The present demonstration of pure epo binding by two different methods suggests that our estimates of the frequencies of cells with receptors are meaningful.

ADDENDUM

The statistical analysis problem presented by the foregoing study is a common one, and its solution is well known. Because the problem involves a comparison of ratios, however, it is not entirely elementary and it is not ordinarily presented in basic texts. This addendum includes a brief presentation of the problem and its solution, using data from one of the experiments for illustration.

The Statistical Model

The data presented in Table III are those summarized in the first line of Table II; of a total of 1428 control cells counted (in 28 distinct fields on a single slide) 11, or 0.77%, were fluorescent. In contrast, of a total of 1785 experimental cells counted (in 24 fields on another single slide), 41 or 2.29% were fluorescent. Because the number of cells counted is a minute fraction of the total number of cells on that slide, the sample proportion of cells that are fluorescent deviates from the overall proportion fluorescent on the slide as a result of sampling fluctuations in the choice of fields to be counted.

The key assumption of this analysis in that each field may be considered as effectively selected at random from all possible fields visualizable on the slide. We need not make any assumption of homogeneity, ie, that every cell has the same probability of being fluorescent, independent of its location on the slide. (The analysis below would remain valid, for example, if regions of low cell density—fewer than 40 cells per field—had a higher or lower proportion of fluorescent cells than regions of high cell density). Although this key assumption is easily falsified, eg, by selecting

TABLE III. Original Data Used to Obtain Mean Values Shown in Table II,Line 1.

Total cells per field		Fluorescent cells per field	
Control	Experimental	Control[a]	Experimental[b]
46	45	1	3
50	89	1	1
51	54	1	0
41	57	0	1
56	83	0	2
72	97	0	4
72	104	0	3
82	119	1	3
60	83	1	0
106	104	0	0
67	87	1	1
46	43	0	2
37	44	0	0
45	90	0	6
41	38	0	0
36	47	0	1
44	54	1	1
55	64	2	2
27	93	0	2
36	92	0	3
34	95	0	3
39	61	0	3
35	64	0	0
56	78	0	0
50		2	
59		0	
42		0	
43		0	
1,428	1,785	11	41
n = 28	n = 24	n = 28	n = 24
$\bar{x}_C = 51.00$	$\bar{x}_E = 74.375$	$\bar{y}_C = 0.3929$	$\bar{y}_E = 1.7083$

[a]Cells incubated with diluent only.
[b]Cells incubated with DACM-epo.

fields which look "typical" in regard to cell number or number of fluorescent cells, discussion of its plausibility under usual laboratory procedures is beyond the scope of this addendum.

A consequence of our key assumption is that the average number of cells counted per field, $\bar{x}$, is an unbiased estimate of the density of cells over the entire slide and that the average number of fluorescent cells per field, $\bar{y}$, is an unbiased estimate of the density of fluorescent cells over the entire slide. Thus, the ratio $p=\bar{y}/\bar{x}$ is a very nearly unbiased estimate of P, the proportion of fluorescent cells on the slide.

The statistical properties of ratio estimators and other nonlinear combinations are discussed in more advanced treatises on statistical methods. A classic reference is Mellor [13]; a more modern source is Kendall and Stuart [14]. Among the most useful of the several approximations available is that called "the law of propagation of

error," or "the delta method," which consists of replacing the nonlinear function by a linear approximation. Thus, we define:

	Illustrative values from control sample of Table II:
n = number of fields examined	28
x_i = number of cells in i^{th} field	
y_i = number of fluorescent cells in i^{th} field	
$\bar{x} = \Sigma x_i/n$ = average number of cells per field	1428/28 = 51.00
$\bar{y} = \Sigma y_i/n$ = average number of fluorescent cells per field	11/28 = 0.39286
$p = \bar{y}/\bar{x}$ = sample proportion of fluorescent cells	$\frac{0.3929}{51.00} = 0.007703 = 0.7703\%$

$$S_x^2 = \frac{\sum(x_i - \bar{x})^2}{n-1} = \text{sample variance of } x = 286.11$$

$$S_y^2 = \frac{\sum(y_i - \bar{y})^2}{n-1} = \text{sample of variance of } y = 0.3955$$

$$s_{xy}^2 = \frac{\sum(x_i - \bar{x})\,(y_i - \bar{y})}{n-1} = \text{sample covariance of } x \text{ and } y = 1.8148$$

The formula for propagation of error for a ratio, here $p = \bar{y}/\bar{x}$, is conveniently expressed as:

$$\frac{SE^2[p]}{p^2} = \frac{SE^2[\bar{y}]}{\bar{y}^2} + \frac{SE^2[\bar{x}]}{\bar{x}^2} - 2\frac{Cov[\bar{x},\bar{y}]}{\bar{x}\,\bar{y}}$$

Where $SE^2[\bar{x}] = s_x^2/n$

$SE^2[\bar{y}] = s_y^2/n$

$Cov[\bar{x},\bar{y}] = s_{xy}/n$

so that SE $[p_c]$, where subscript c refers to control, can be obtained by solving the equation,

$$\frac{SE^2[p_c]}{(0.007703)^2} = \frac{0.3955/28}{(0.39286)^2} + \frac{286.22/28}{(51.00)^2} - 2\frac{1.8148/28}{(0.39286)\,(51.00)} = 0.08899$$

we find that $SE[p_c] = 0.002316$ (or 0.2316%) and
in similar fashion we find, upon analysis of the experimental samples (subscript E),

$P_E = 0.22971$ or 2.297% and $SE[p_E] = 0.00399$ (or 0.399%)
To test the statistical significance of the difference we compute,

$$\bar{z} = \frac{p_E - p_c}{(SE^2\,[p_E] + SE^2\,[p_c])^{1/2}} = \frac{2.297 - 0.399}{(0.232^2 + 0.399^2)^{1/2}} = \frac{1.527}{0.461} = 3.3$$

which is, indeed, highly significant.

A Simpler, But More Risky Analysis

If, in place of our key assumption (fields effectively random on the slide), we are prepared to make the far stronger assumption of homogeneity (all areas of the slide are equivalent, in that the chance of being fluorescent is identical for every cell, regardless of position on the slide) then we may regard p as a simple binomial variable (expressed as percent) and calculate the standard error according to the elementary formula,

$$SE\,[p] = \frac{[(p)\,(100\text{-}p)]^{1/2}}{\text{no. cells}} = \frac{[0.7703 \times 99.2297]^{1/2}}{1428} = 0.2314\%$$

which, in this example, is very close to the result (0.2316%) obtained with the far more broadly applicable method given in the previous section. In practice, the two methods will give nearly the same results except in cases where examination of the data shows clear evidence of variability between fields in proportion fluorescent. However, judgments of the magnitude of such variability can be highly subjective and, despite its greater computational complexity, the method of the previous section is to be preferred in general.

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