From www.bloodjournal.org by guest on October 30, 2017. For personal use only.

Effects of Butyrate on the Erythropoietin Receptor of Cell Line IW201

By Olivier Hermine, Yi Jie Dong, and Eugene Goldwasser

The murine erythroleukemic cell line, IW201, normally expresses only low-affinity erythropoietin receptors. Exposure of these cells for 48 hours to sodium butyrate results in a change in receptor kd from about 600 pmol/L to 100 to 200 pmol/L. This change in affinity is accompanied by downregulation of both receptor number and receptor mRNA. Cells exposed to sodium butyrate for 2 hours show a similar change in kd but no change in receptor number. The butyrate effect on kd at 2 hours is abrogated by either cycloheximide or actinomycin D. These data indicate that an accessory protein induced by sodium butyrate is responsible for high-affinity binding of erythropoietin.

© 1994 by The American Society of Hematology.

ERYTHROPOIETIN (epo), the primary regulator of erythropoiesis, acts via specific receptors (epo-R) on erythroid progenitor cells.[1] Though human and murine epo-R cDNAs and genes have been cloned,[2-5] little is known about the higher-order molecular structure or signal transduction mechanisms of the epo-R. Specific binding of epo has been found on cells of erythroid lineages, including erythroid progenitor spleen cells of mice infected by the Friend virus,[6] murine[7,8] and human erythroleukemias,[9] and cells from fetal liver,[10] among others.

The number of receptors per cell is variable, being in the range of 20 to 1,000 per cell. Two affinity classes of the epo-R have been found,[6] one with apparent kd values of 300 to 700 pmol/L and one of higher affinity with kd values in the range of 10 to 100 pmol/L. In general, cells in which epo affects both proliferation and differentiation have both classes of receptor, whereas cells that respond only by growth or that do not respond (such as MEL cells) have only low-affinity epo-Rs. The murine epo-R cDNA derived from an MEL cell library when transfected into transformed monkey kidney cells (COS) effects the expression of both low- and high-affinity receptors.[2] These results suggest that a second subunit or an accessory protein, endogeneous to COS cells, confers high affinity on the transfected low-affinity epo-R. Recent results from this laboratory confirmed the existence, in Chinese hamster ovary cells, of an accessory factor that can convert low-affinity epo-R to high affinity.[11]

The erythroleukemic cell line IW201 used in this study was isolated from the spleens of mice injected with the complete Friend murine leukemia virus (anemia strain).[12] These cells have the phenotype of proerythroblasts but are not responsive to epo although they express low-affinity receptors. Exposure to butyrate or hemin causes IW201 cells to synthesize increased α-globin mRNA but not β-globin mRNA or hemoglobin.[13]

In this report we confirm that IW201 cells have epo receptors of low affinity. We also show that butyrate causes downregulation of both the epo-R, and its mRNA, and a switch of receptor affinity from low to high affinity. This change in affinity is dependent on transcription and protein synthesis, and appears to be caused by a new protein expressed in the induced cells. We suggest that this new protein is the accessory protein inferred from the earlier experiments.

MATERIALS AND METHODS

Cell culture. IW201 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Sigma Chemicals, St Louis, MO) supplemented with 5% fetal calf serum (GIBCO, Grand Island, NY). Cells were seeded at 2×10^5 cells per mL in Nunc T-25 flasks (Scientific Supply, Schiller Park, IL) and passed every 5 to 6 days. Sodium butyrate (ICN Pharmaceuticals Inc, Plainview, NY) where present was added to a final concentration of 2 mmol/L. Cycloheximide and actinomycin D (both from Sigma) where present were added to a final concentration of 100 μmol/L and 4 μmol/L, respectively. These additions were made 3 hours before 2 mmol/L sodium butyrate was added to the cells.

Radioiodination of epo. Radioiodination was performed according to Fracker and Speck.[14] In brief, 0.5 μg of human recombinant epo (Amgen, Inc, Thousand Oaks, CA) and 200 μCi of $Na^{125}I$ (Amersham Corp, Arlington Heights, IL) were added to a micro sample vial (American Scientific Products, McGaw Park, IL) coated with 10 μg of Iodo-gen (Pierce Chemical Co, Rockford, IL). The mixture was left at 22°C for 30 seconds, then transferred to a tube containing 200 μL of KI (10 mg/mL in 0.5 phosphate buffer pH 7.0) and 300 μL of column buffer (0.1 mol/L phosphate, 0.1 mol/L NaCl, 0.05% gelatin) with 3 mmol/L sodium thiosulfate, and ^{125}I-epo was separated from free iodide on a G-25 column. The specific activity of the iodinated epo was 50 μCi/μg, equivalent to 2 atoms of iodine per epo molecule.

Binding assay. Equilibrium binding of epo to IW201 cells was performed in triplicate, in polypropylene tubes (5 mL) containing 3 to 5×10^6 cells, 0.01 to 2.0 nmol/L ^{125}I-epo, with or without a 100-fold excess of unlabeled epo. The incubation medium was DMEM and 1% bovine serum albumin, and the total volume was 100 μL. Samples were incubated for 1 hour at 37°C with intermittent shaking. The whole sample was transferred to a 1.5-mL Eppendorf tube containing 0.5 mL of dibutylphthalate, centrifuged for 1 minute at 15,000*g*, and immediately frozen in liquid nitrogen. The tip with the cell pellet was cut off and radioactivity was determined in a gamma counter. Specific binding was defined as the amount of labeled epo displaceable by a 100-fold excess of unlabeled epo. Nonspecific binding ranges from 10% to 45% of total binding depending on the experiment. Within a single experiment it varies about threefold. Apparent kd values and X-intercepts were determined by regression analysis of the Scatchard plots.

The use of 37°C without azide was justified by our measurement of internal versus external epo after 1 hour of incubation. The cells were incubated with ^{125}I-epo for 1 hour and then washed with 3% NaCl at pH 3.0 for 1 minute to remove epo that had not been

From the Department of Biochemistry and Molecular Biology, The University of Chicago, Chicago, IL.

Submitted November 15, 1993; accepted April 6, 1994.

Supported in part by Grant No. HL21676 from the National Heart, Lung, and Blood Institute.

Address reprint requests to Eugene Goldwasser, PhD, Department of Biochemistry and Molecular Biology, The University of Chicago, 920 E 58th St, Chicago, IL 60637.

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. section 1734 solely to indicate this fact.

© 1994 by The American Society of Hematology.

0006-4971/94/8403-0027$3.00/0

From www.bloodjournal.org by guest on October 30, 2017. For personal use only.

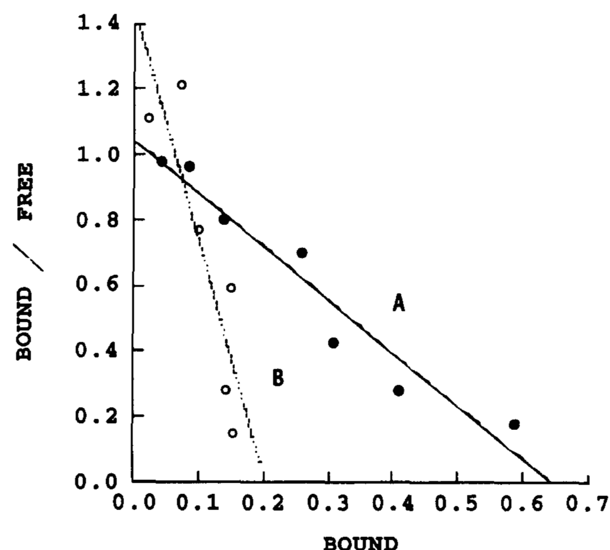

Fig 1. Effect of butyrate on receptor binding of epo. Scatchard plot of binding data using IW201 cells. (A) (●) Control cells; (B) (○) cells exposed to 2 mmol/L sodium butyrate for 48 hours. Bound refers to specific binding in femtomoles.

internalized, and cellular radioactivity was measured. We found that cells washed at low pH contained about 7% of the radioactivity found in control cells.

RNA isolation and Northern blot analysis. Total cellular RNA was isolated by the method of Chirgwin et al[15] and electrophoresed on 1% agarose (Bethesda Research Laboratories, Gaithersburg, MD) gel containing 2.2 mol/L formaldehyde. The size-fractionated RNA was then transferred to GeneScreenPlus filters (E.I. du Pont de Nemours & Co, Inc, Wilmington, DE). Murine epo-R and actin or hexokinase mRNAs were detected by hybridization to nick-translated probes prepared by using ^{32}P dCTP (400 Ci/mmol) and a nick-translation kit, both from Amersham.

RESULTS

In a large number of experiments we have amply confirmed the finding that IW201 cells express only low-affinity receptors binding ^{125}I-epo with kd values ranging from 300 to 800 pmol/L, with about 300 to 400 receptors per cell.[11] A typical Scatchard plot of IW201 binding is shown in Fig 1A; in this experiment the regression line yields an apparent kd of 620 pmol/L (95% confidence limits 480 to 880) and 370 receptors per cell (the regression coefficient R was .97) in agreement with our earlier findings.[11]

Binding characteristics of IW201 cells incubated in the presence of 2 mmol/L sodium butyrate for 48 hours were studied. The Scatchard plot (Fig 1B) shows a marked decrease in the number of receptors (100 per cell). Surprisingly, the affinity of the epo-R was altered so that there was a single class of higher affinity receptors with an apparent kd of 120 pmol/L (95% confidence limits 75 to 330, R = .88).

This finding raised the possibility of a direct effect of butyrate on the cell membrane, which might explain the change of affinity. Therefore, we repeated the same experiment, but after only 2 hours of incubation. The Scatchard plot from binding data is shown in Fig 2; the number of receptors did not change significantly, being 290 per cell. In contrast, the affinity of the receptor was increased, with an apparent kd of 120 pmol/L (95% confidence limits 150 to 350, R = .92), in contrast to the control cells.

Although the fact that butyrate was able to cause a change of epo-R affinity within 2 hours might suggest a direct effect on the existing receptors, an indirect effect due to the synthesis of a new protein induced by butyrate could not be ruled out. Indeed, it has been shown that butyrate is able to induce the synthesis of new transcripts in rat islet cells within 30 minutes.[16] Therefore, we repeated the same experiment in the presence of actinomycin D or cycloheximide. The Scatchard plots from a typical experiment are shown in Fig 2. Both inhibitors blocked the appearance of higher-affinity receptors. The cells treated with both butyrate and cycloheximide had 340 receptors per cell with an apparent kd of 410 pmol/L (95% confidence limits of 350 to 490, R = .99). Those treated with butyrate and actinomycin D had 610 receptors per cell with an apparent kd of 850 pmol/L (95% confidence limits of 810 to 870, R = .99). The data from representative experiments are summarized in Table 1. These data argue strongly for the requirement for new synthesis of mRNA and protein preceeding the change of affinity during butyrate induction.

epo-R mRNA expression during erythroid differentiation. Because of the significant decrease in receptor number after butyrate treatment we examined the expression of the epo-R mRNA in these cultured IW201 cells. The cells were cultured for periods from 1 to 24 hours with or without 2 mmol/L sodium butyrate, RNA extracted, and the relative amounts of epo-R mRNA evaluated by the Northern blot

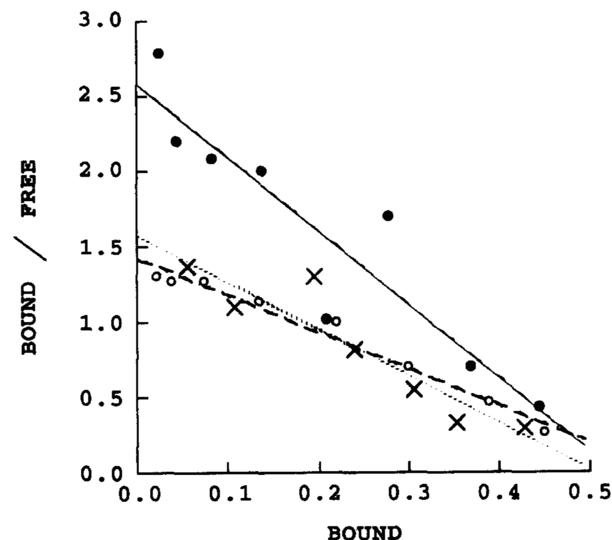

Fig 2. Abrogation of butyrate effect on binding by cycloheximide and actinomycin. (●) Binding by IW201 cells after 2 hours of exposure to 2 mmol/L sodium butyrate; (○) cells treated with 100 μmol/L cycloheximide for 3 hours before sodium butyrate (2 mmol/L) was added; (×) cells treated with 4 μmol/L actinomycin D for 3 hours before butyrate was added. Bound refers to specific binding in femtomoles.

From www.bloodjournal.org by guest on October 30, 2017. For personal use only.

method. The results (Fig 3) show a definite decrease in epo-R mRNA by 6 hours when the control actin mRNA was unchanged. By 12 hours and later both epo-R and actin mRNAs were decreased. Because of the change in actin mRNA, we repeated the experiment using hexokinase mRNA to normalize the Northern blot. It too decreased during the first 24 hours of incubation of the cells in the presence of butyrate but not after that (data not shown). The epo-R mRNA decreased in the next 24 hours when the hexokinase mRNA did not change. These data indicate that the downregulation of epo-R because of butyrate is at least in part at the mRNA level. The cells respond to butyrate with respect to the lower affinity epo-R by decreased expression while increasing the expression of the accessory protein that increases the affinity of the receptor.

DISCUSSION

There is, of course, precedent for the role of accessory proteins in increasing the affinity of receptors as shown for the receptors for the interleukins (IL)-2,[17] -3,[18] -5,[19] and -6[20] and granulocyte-macrophage colony-stimulating factor.[21] In the case of IL-6 the accessory protein analagous to the one we describe here was found to be the glycoprotein gp130. We have used a probe for that gene product and found no signal by Northern blot in RNA from butyrate induced IW201 cells (data not shown), suggesting that the signal-transducing protein, gp130, which converts the low-affinity IL-6 receptor to high affinity is not responsible for the butyrate-induced high-affinity epo-R. In a recent report Nagao et al[22] showed that tunicamycin treatment of cells expressing both low- and high-affinity epo-R results in expression of only high-affinity receptors. These investigators suggest that an accessory protein is responsible for this effect.

We conclude from these observations that in IW201 cells sodium butyrate causes the induction of a new protein that alters the affinity of the epo-R to that approximating the kd found on cells that differentiate in response to epo. This induction is fairly rapid and is followed by downregulation of the expression of the epo-R. We do not know whether there is any causal relationship between these two effects of sodium butyrate. The induction, in IW201 cells, of the putative accessory protein by sodium butyrate may, by use of subtractive hybridization, provide a method to clone the putative accessory protein. These experiments are now in progress.

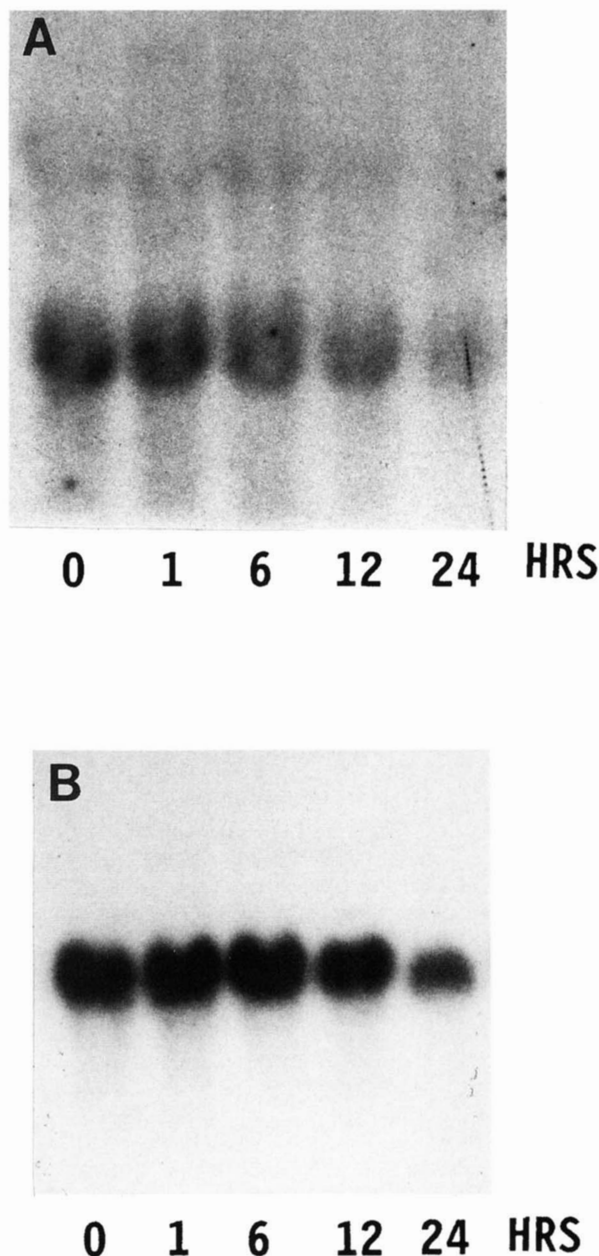

Fig 3. Butyrate causes downregulation of epo-R mRNA. (A) RNA from IW201 cells was extracted at 0, 1, 6, 12, and 24 hours after addition of sodium butyrate. The Northern blot was probed with end-labeled epo-R cDNA. (B) The same blot was stripped and probed with an actin cDNA.

Table 1. Effect of Butyrate on the Affinity of the epo-R

	kd (pmol/L)	95% Confidence Limits	R*	Receptors/ Cells
Control	620	480-880	.97	370
Butyrate treatment 48 h	120	75-330	.88	100
Butyrate treatment 2 h	210	150-350	.92	290
Butyrate 2 h + cycloheximide	410	350-490	.99	340
Butyrate 2 h + actinomycin	850	810-870	.99	610

* R is the correlation coefficient for the regression fit of the data.

ACKNOWLEDGMENT

We are indebted to Dr Alan D'Andrea for the epo receptor cDNA, Dr Elaine Fuchs for the actin probe, Dr Graeme Bell for the hexokinase probe, Dr T. Kishimoto for the gp130 probe, and Dr Bruno Varet for the IW201 cell line.

From www.bloodjournal.org by guest on October 30, 2017. For personal use only.

REFERENCES

1. Krantz SB, Goldwasser E: Specific binding of erythropoietin to spleen cells infected with the anemia strain of Friend virus. Proc Natl Acad Sci USA 81:7574, 1984

2. D'Andrea AD, Lodish HF, Wong GG: Expression cloning of the murine erythropoietin receptor. Cell 57:277, 1989

3. Winkelmann JC, Penny LA, Deaven LL, Forget BG, Jenkins RB: The gene for the human erythropoietin receptor: Analysis of the coding sequence and assignment to chromosome 19P. Blood 76:24, 1990

4. Jones SS, D'Andrea AD, Haines LL, Wong GG: Human erythropoietin receptor: Cloning, expression and biological characterization. Blood 76:31, 1990

5. Kuramochi S, Ikawa Y, Todokoro K: Characterization of murine erythropoietin receptor genes. J Mol Biol 216:567, 1990

6. Sawyer ST, Krantz SB, Goldwasser E: Binding and receptor-mediated endocytosis of erythropoietin in Friend virus-infected erythroid cells. J Biol Chem 262:5554, 1987

7. Mayeux P, Billat C, Jacquot R: Murine erythroleukemia cells (Friend cells) possess high affinity binding sites for erythropoietin. FEBS Lett 211:229, 1987

8. Sasaki R, Yamagawa S, Hitoma K, Chiba H: Characterization of erythropoietin receptor on murine erythroid cells. Eur J Biochem 168:43, 1987

9. Broudy VC, Lin N, Egrie J, DeHaven C, Weiss T, Papayannopoulou T, Adamson JW: Identification of the receptor for erythropoietin in human and murine erythroleukemia cells and modulation by phorbol ester and dimethylsulfoxide. Proc Natl Acad Sci USA 85:6513, 1988

10. Fukamachi H, Saito T, Tojo A, Kitamura T, Urabe A, Takaku F: Binding of erythropoietin to CFU-E derived from fetal mouse liver cells. Exp Hematol 15:833, 1987

11. Dong YJ, Goldwasser E: Evidence for an accessory component that increases the affinity of the erythropoietin receptor. Exp Hematol 21:483, 1993

12. Choppin J, Casadevall N, Lacombe C, Wendling F, Goldwasser E, Berger R, Tambourin P, Varet B: Production of erythropoietin by cloned malignant murine erythroid cells. Exp Hematol 13:610, 1985

13. Beru N, Maples PB, Hermine O, Goldwasser E: Differential expression of α- and β-globin genes in erythroleukemic cell lines. Mol Cell Biol 10:3591, 1990

14. Fracker PH, Speck JC Jr: Protein and cell membrane iodination with a sparingly soluble chloroamide, 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril. Biochem Biophys Res Commun 80:849, 1978

15. Chirgwin JM, Przybyla AE, McDonald RJ, Rutter WJ: Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18:5294, 1979

16. Streutker C, Drucker DJ: Rapid induction of parathyroid hormone-like peptide gene expression by sodium butyrate in a rat islet cell line. Mol Endocrinol 5:703, 1991

17. Voss SD, Leary TP, Sondel PM, Robb RJ: Identification of a direct interaction between interleukin 2 and the p64 interleukin 2 receptor γ chain. Proc Natl Acad Sci USA 90:2428, 1993

18. Kitamura T, Sato N, Arai K, Miyajima A: Expression cloning of the human IL-3 receptor cDNA reveals a shared β subunit for the human IL-3 and GM-CSF receptors. Cell 66:1165, 1991

19. Devos R, Plaetinck G, Van der Heyden J, Cornelis S, Vanderkerckhove J, Fiers W, Tavernier J: Molecular basis of a high affinity murine interleukin-5 receptor. EMBO J 10:2135, 1991

20. Hibi M, Murakami M, Saito M, Hirano T, Taga T, Kishimoto T: Molecular cloning and expression of an IL-6 signal transducer, gp 130. Cell 63:1149, 1990

21. Hayashida K, Kitamura T, Gorman DM, Arai K, Yokota T, Miyajima A: Molecular cloning of a second subunit of the receptor for human granulocyte-macrophage colony-stimulating factor (GM-CSF): Reconstitution of a high-affinity GM-CSF receptor. Proc Natl Acad Sci USA 87:9655, 1990

22. Nagao M, Matsumoto S, Masuda S, Sasaki R: Effect of tunicamycin treatment on ligand binding to the erythropoietin receptor: Conversion from two classes of binding sites to a single class. Blood 81:2503, 1993

From www.bloodjournal.org by guest on October 30, 2017. For personal use only.

blood

1994 84: 811-814

Effects of butyrate on the erythropoietin receptor of cell line IW201

O Hermine, YJ Dong and E Goldwasser

Updated information and services can be found at:
http://www.bloodjournal.org/content/84/3/811.full.html

Articles on similar topics can be found in the following Blood collections

Information about reproducing this article in parts or in its entirety may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#repub_requests

Information about ordering reprints may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#reprints

Information about subscriptions and ASH membership may be found online at:
http://www.bloodjournal.org/site/subscriptions/index.xhtml

Blood (print ISSN 0006-4971, online ISSN 1528-0020), is published weekly by the American Society of Hematology, 2021 L St, NW, Suite 900, Washington DC 20036.
Copyright 2011 by The American Society of Hematology; all rights reserved.