hydroxyestrone infusion produced no decrease in serum prolactin, with the apparent increase being not significant by analysis of variance. We do not know why we failed to obtain a response in these four studies, but it is noteworthy that the two responding subjects who were studied repeatedly exhibited either a total or partial prolactin suppression in each study, whereas the one nonresponding subject twice failed to respond in both studies, suggesting that the prolactin response to 2-hydroxyestrone is subject and not time dependent.

The profound suppression by 2-hydroxyestrone of prolactin secretion in young women has significant physiological and clinical implications. The infusion of 2-hydroxyestrone resulted in blood concentrations of 2-hydroxyestrone that were six times that at time 0 (90 versus 15 pg/ml) as determined by radioimmunoassay (*15*), indicating a very rapid metabolic clearance of the administered material. The physiological relevance of this observation must be considered in light of the demonstrated capacity of neuronal tissue to convert estrogens to 2-hydroxyestrone (*16–18*). The material biosynthesized in situ would then be expected to affect the neuronal target tissue at physiological concentrations.

Regulation of prolactin secretion by estrogens in the human can be construed as one of dual nature, with estradiol acting in a positive feedback mode and its 2-hydroxylated metabolite acting in an inhibitory fashion. The control of prolactin secretion by estrogens would then be dependent on the activity of the estradiol-2-hydroxylase enzyme in the appropriate central or pituitary sites. Evidence has been obtained that opiate agonists that stimulate prolactin release (*19*) are effective inhibitors of estradiol-2-hydroxylase in the rat brain, whereas the opiate antagonist naloxone, which suppresses prolactin release, increases estradiol-2-hydroxylase activity in the brain (*20*). The action of opiate agonists and antagonists on prolactin release is therefore in concert with their impact on estradiol-2-hydroxylase in the brain, which suggests that the action of the opiates on prolactin release is modulated by their effect on estrogen metabolism in neuronal tissue.

The suppression of prolactin secretion by 2-hydroxyestrone may have significant clinical applications. Hyperprolactinemia has now been associated with reproductive disorders in both men and women (*21*), and the suppression of pituitary prolactin secretion has become an important treatment. The suppression of prolactin secretion by a nonuterotropic natural estrogen may offer an alternative and possibly superior treatment of hyperprolactinemia.

It is not known whether the inhibition of prolactin secretion by 2-hydroxyestrone occurs at the pituitary or at the hypothalamic level, nor is it known whether dopaminergic mechanisms are involved. Resolution of some of these questions may make it possible to use the effect of 2-hydroxyestrone on serum prolactin to differentiate between functional hyperprolactinemia and that resulting from the presence of pituitary tumors, a goal which has thus far proved elusive (*22*).

J. FISHMAN

Rockefeller University, New York 10021

D. TULCHINSKY

Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts 02115

References and Notes

1. S. S. C. Yen, Y. Ehara, T. M. Siler, *J. Clin. Invest.* **53**, 652 (1974).
2. A. G. Frantz, D. L. Kleinberg, G. L. Noel, *Recent Prog. Horm. Res.* **28**, 455 (1976).
3. Y. Ehara, T. M. Siler, S. S. C. Yen, *Am. J. Obstet. Gynecol.* **125**, 455 (1976).
4. J. E. Tyson and H. G. Friesen, *ibid.* **116**, 377 (1973).
5. S. K. Quadi, T. Oyama, H. G. Spies, *Endocrinology* **104**, 1649 (1979).
6. C. Martucci and J. Fishman, *ibid.* **101**, 1709 (1977).
7. J. H. Clark, Z. Paszko, E. J. Peck, *ibid.* **100**, 91 (1977).
8. J. Fishman, *J. Clin. Endocrinol. Metab.* **23**, 207 (1963).
9. P. Ball, H. P. Gelbke, R. Knuppen, *ibid.* **40**, 406 (1975).
10. F. Naftolin, H. Morishita, I. J. Davies, R. Todd, K. J. Ryan, J. Fishman, *Biochem. Biophys. Res. Commun.* **64**, 905 (1975).
11. V. Gethman and R. Knuppen, *Hoppe-Seylers Z. Physiol. Chem.* **357**, 1011 (1976).
12. E. Y. Adashi, J. Rakoff, W. Divers, J. Fishman, S. S. C. Yen, *Life Sci.* **25**, 2051 (1979).
13. J. S. Schinfeld, D. Tulchinsky, I. Schiff, J. Fishman, *J. Clin. Endocrinol. Metab.* **50**, 408 (1980).
14. P. Hwang, H. Guyda, H. Friesen, *Proc. Natl. Acad. Sci. U.S.A.* **68**, 1902 (1971).
15. I. Yoshizawa and J. Fishman, *J. Clin. Endocrinol. Metab.* **32**, 3 (1971).
16. J. Fishman and B. Norton, *Endocrinology* **96**, 1054 (1975).
17. J. Fishman, F. Naftolin, T. J. Davies, K. J. Ryan, Z. Petro, *J. Clin. Endocrinol. Metab.* **42**, 177 (1976).
18. S. M. Paul and J. Axelrod, *Endocrinology* **101**, 1604 (1977).
19. M. S. Gold, D. E. Redmond, R. F. Donabedian, *ibid.* **105**, 284 (1979).
20. J. Fishman, B. Norton, E. Hahn, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 2574 (1980).
21. A. G. Frantz, *N. Engl. J. Med.* **298**, 201 (1978).
22. C. Fenari, P. G. Grosignani, R. Coldara, M. C. Piccioti, A. Malinverni, B. Barattini, P. Rambini, P. Tellol, *J. Clin. Endocrinol. Metab.* **50**, 23 (1980).
23. This research was supported by grant CA 22795 from the National Cancer Institute.

11 March 1980; revised 28 May 1980

Erythroid Differentiation of Clonal Rauscher Erythroleukemia Cells in Response to Erythropoietin or Dimethyl Sulfoxide

Abstract. *Clonal lines of Rauscher erythroleukemia cells exhibited selective responses to two inducers of differentiation, erythropoietin and dimethyl sulfoxide. There were substantial quantitative differences between clones that responded to both inducers. Several clones differentiated only in response to erythropoietin. Erythropoietin stimulated cell proliferation and differentiation whereas dimethyl sulfoxide inhibited proliferation, suggesting dissimilar modes of action.*

A definitive understanding of the control of erythroid differentiation requires that the mechanism of action of erythropoietin (*1*) be clarified. Advances in our knowledge of erythropoiesis have been made with Friend erythroleukemia cells—a murine viral leukemia line, grown in continuous culture, that exhibits erythroid differentiation in response to various chemical inducers (*2*). However, this cell line does not respond to erythropoietin. Hence, studies of the hormone's mechanism of action have been limited to examination of its effects on unfractionated bone marrow (*3*) or mouse fetal liver (*4*), both of which are heterogeneous (although the fetal liver is much less so). Clearly, a cell line that could be maintained continuously in vitro and that differentiated selectively in response to erythropoietin would provide an ideal system for studying the hormone's biochemistry.

Such a system is provided by the recently described Rauscher murine erythroleukemia line (*5*). The parent, uncloned cell line undergoes erythroid differentiation in response to either erythropoietin or dimethyl sulfoxide (DMSO). We maintained the cells in plastic culture flasks or petri dishes in continuous suspension culture in a mixture of Eagle's minimum essential medium (Dulbecco's modification) and fetal calf serum (10 percent) in a humidified atmosphere of 95 percent air and 5 percent CO_2 (37°C). Individual cells gave rise to discrete colonies when grown in plasma-clot culture (*6*), a semisolid medium (100 cells per 0.1-ml clot). The clots were fixed on glass slides with glutaraldehyde, stained with dimethoxybenzidine and H_2O_2 to detect hemoglobin, and counterstained with hematoxylin. The cells in each colony were then examined microscopically for erythroid

0036-8075/80/1003-0074$00.50/0

differentiation. After 5 days in plasma-clot culture in the absence of inducer (erythropoietin or DMSO), distinct colonies formed containing 16 to 128 rather uniform cells with large nuclei (Fig. 1A). Hemoglobin was detectable in only 1 to 2 percent of the colonies, and in only a few cells. In contrast, when grown in the presence of erythropoietin, the colonies were larger, containing 32 to over 300 cells, strongly suggesting that the hormone stimulates a proliferative response. Moreover, 40 to 60 percent of the colonies contained 8 to 64 hemoglobinized cells, some without nuclei, indicative of differentiation following proliferation (Fig. 1B). Dimethyl sulfoxide also induced differentiation in the primary line, as evidenced by the development of colonies of 4 to 32 hemoglobinized cells, but the small size of these colonies indicates inhibition of cell proliferation (Fig. 1C).

We cloned the primary Rauscher erythroleukemia line by dilutional plating techniques (7) in order to derive lines with great genetic homogeneity and favorable biological properties—in particular, a selective response to erythropoietin or DMSO. We assessed the response of more than 30 clones to the two inducers in plasma-clot cultures (Table 1). Many of the clones responded to both erythropoietin and DMSO and exhibited substantial quantitative differences. Several clones differentiated only in response to erythropoietin.

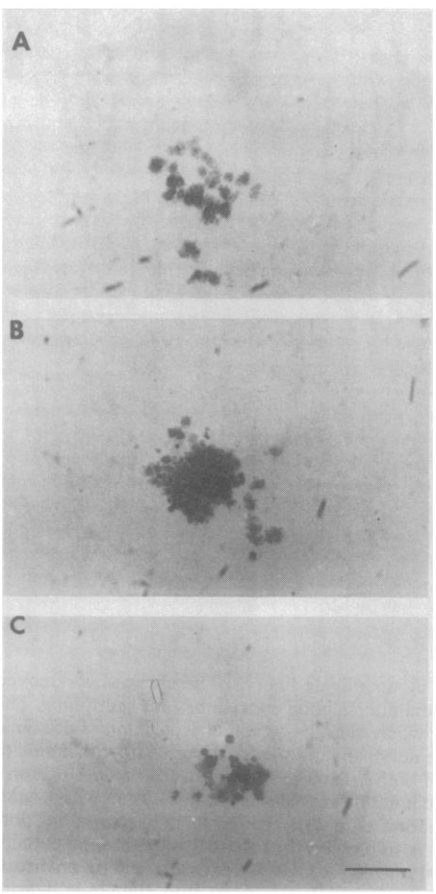

Fig. 1. Colonies of Rauscher erythroleukemia cells incubated in plasma-clot culture with (A) no inducer, (B) erythropoietin (1 U/ml) (8), or (C) 0.5 percent DMSO. Scale bar, 50 μm.

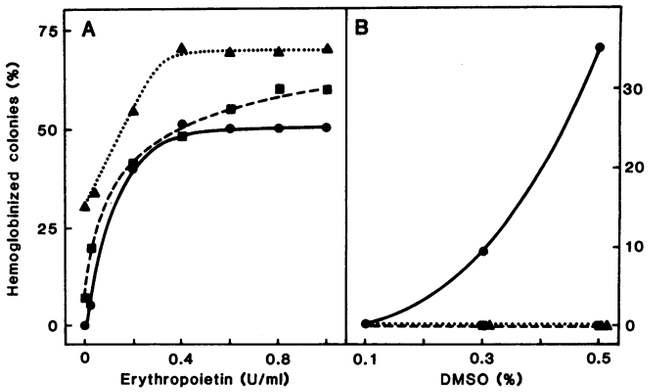

Fig. 2. Effects of different concentrations of erythropoietin (A) or DMSO (B) on differentiation of Rauscher erythroleukemia clones R-19 (●), R-31 (■), and R-37 (▲) in plasma-clot culture.

An examination of the dose-response relations for erythropoietin and DMSO of various clones reveals these differences (Fig. 2, A and B). Clones R-19, R-31, and R-37 formed colonies 0, 7, and 30 percent of which were positive for hemoglobin, respectively, without added erythropoietin. All three responded to the hormone and reached maxima of 60, 61, and 75 percent positive for hemoglobin, respectively. The relatively high degree of "spontaneous" differentiation shown by clone R-37 may have been due to increased sensitivity to erythropoietin in the fetal calf serum used in the growth medium, a conclusion supported by the leftward shift of the dose-response curve for the clone relative to those for R-19 and R-31. Erythropoietin also increased the plating efficiencies of these clones two- to sevenfold (from 7 to 10 percent to 20 to 60 percent), another indication of its growth-promoting action. Only R-19 differentiated in response to DMSO; 30 percent of the R-19 colonies were positive for hemoglobin at 0.5 percent DMSO. The plating efficiences of all three clones were decreased by DMSO. An increase in DMSO to 0.9 percent resulted in no colony formation by any of the three clones.

Preliminary studies show a nearly simultaneous increase in DNA, RNA, and protein synthesis in clone R-19 (over control and DMSO values) 6 to 12 hours after exposure to erythropoietin. This is consistent with the observed proliferative response, and strongly suggests that erythropoietin and DMSO induce differentiation by dissimilar mechanisms.

Thus we have begun to distinguish between the cellular responses to erythropoietin, which is operative in nature, and those to DMSO, which has been employed so extensively in the study of Friend erythroleukemia cells. Perhaps most striking is the observation that in addition to induction of differentiation, both inducers affect cell proliferation: erythropoietin increases it whereas DMSO decreases it. Erythroid cells also proliferate in response to erythropoietin

Table 1. Erythroid differentiation of Rauscher erythroleukemia clones in plasma-clot culture (100 cells per 0.1-ml clot). The cells were incubated in the absence or presence of erythropoietin (1 U/ml) or 0.5 percent DMSO for 5 days.

Clone	Hemoglobinized colonies (%)			Plating efficiency (%)
	Control	Erythropoietin	DMSO	
R-1	0	60	0	20
R-2	2	70	2	14
R-3	22	38	50	8
R-4	0	0	0	8
R-5	15	82	50	12
R-6	0	10	0	30
R-7	4	79	50	10
R-8	0	65	0	2
R-10	0	86	0	6
R-11	12	69	0	6
R-12	25	33	33	4
R-13	8	46	28	32
R-14	0	46	41	6
R-15	10	67	0	6
R-17	0	86	41	16
R-19	0	58	35	10
R-20	0	44	0	8
R-22	86	100	67	4
R-23	5	40	58	10
R-24	0	48	58	8
R-25	0	79	29	4
R-28	32	58	38	8
R-29	24	67	25	6
R-31	7	61	0	6
R-33	0	49	21	10
R-34	4	46	18	8
R-35	0	0	0	6
R-37	31	71	0	8
R-38	0	86	11	10
R-39	0	11	0	4
Primary line	0	46	28	16

in vivo; hence, the observations in vitro presumably reflect the same mechanism. In assessing the mode of action of erythropoietin, therefore, it is necessary to understand this proliferative response and to ask whether differentiation into enucleate, hemoglobin-rich erythroid cells is the end of a single program, the expression of which is due to one hormone-cell interaction, or whether the proliferative and maturational responses to erythropoietin are due to discrete signals that may be characterized and, ultimately, experimentally controlled. The availability of the clonal lines described here now permits an examination of this question.

ARTHUR J. SYTKOWSKI*
AUGUST J. SALVADO†
GEORGIANNA M. SMITH
CAROL J. MCINTYRE
Division of Hematology and Oncology, Children's Hospital Medical Center, and Sidney Farber Cancer Institute, Department of Pediatrics, Harvard Medical School, Boston, Massachusetts 02115
N. J. DEBOTH
Department of Experimental Pathology, Medical Faculty, Erasmus University, Rotterdam, Netherlands

References and Notes

1. A. J. Sytkowski, *Biochem. Biophys. Res. Commun.* **93**, 354 (1980); T. Miyake, C. K. H. Kung, E. Goldwasser, *J. Biol. Chem.* **252**, 5558 (1977).
2. C. Friend, W. Scher, J. G. Holland, T. Sato, *Proc. Natl. Acad. Sci. U.S.A.* **68**, 378 (1971); U. Nudel, J. E. Salmon, M. Terada, A. Bank, R. A. Rifkind, P. A. Marks, *ibid.* **74**, 1100 (1977); J. Gusella, R. Geller, B. Clarke, V. Weeks, D. Housman, *Cell* **9**, 221 (1976); H.-Y. Y. Hu, J. Gardner, P. Aisen, A. I. Skoultchi, *Science* **197**, 559 (1977); B. P. Alter and S. C. Goff, *ibid.* **207**, 647 (1980); A. Leder and P. Leder, *Cell* **5**, 319 (1975); S. H. Orkin, D. Swan, P. Leder, *J. Biol. Chem.* **250**, 8753 (1975).
3. M. Gross and E. Goldwasser, *Biochemistry* **8**, 1795 (1969).
4. J. Paul and J. A. Hunter, *J. Mol. Biol.* **42**, 31 (1969); M. Djaldetti, H. Preisler, P. A. Marks, R. A. Rifkind, *J. Biol. Chem.* **247**, 731 (1972).
5. N. J. deBoth, M. Vermey, E. van't Hull, E. Klootwijk-van-Dijke, L. J. L. D. van Griensven, L. N. M. Mol, T. J. Stoof, *Nature (London)* **272**, 626 (1978).
6. D. L. McLeod, M. M. Shreeve, A. A. Axelrad, *Blood* **44**, 517 (1974).
7. J. A. Robb, *Science* **170**, 857 (1970).
8. For these studies we used human urinary erythropoietin prepared from an anemic donor. The specific activity in vitro was measured at 20 U/mg by a mouse bone marrow technique, with NIH lot M-7-TaLSL used as a standard. Rauscher erythroleukemia cells also respond to human erythropoietin of higher specific activity and to sheep plasma erythropoietin (Connaught).
9. We thank D. G. Nathan for constant encouragement and helpful discussions and C. Lewis for expert secretarial assistance. This work was supported in part by PHS grants CA-18662 and CA-26105 and by a Medical Foundation fellowship (A.J.S.).

* To whom correspondence should be addressed.
† Visiting research associate, Harvard Medical School; permanent address: Department of Hematology, Walter Reed Army Institute of Research, Washington, D.C. 20012.

4 April 1980; revised 9 June 1980

Opioid Receptors Undergo Axonal Flow

Abstract. *Previous studies have indicated the presence of opiate receptors on axons of the rat vagus nerve and on other small diameter fibers. In examinations of the effect of ligation on the distribution of receptors in the vagus nerve by in vitro labeling light microscopic autoradiography, a large buildup of receptors was found proximal to the ligature. This result indicates an axonal flow of receptors.*

Axoplasmic flow and axonal and dendritic transport are responsible for delivery of various macromolecules to distant parts of the neuron (*1*). Although the mechanisms of these transports are unknown, their properties have been studied (*1*). We report evidence that receptors undergo such movement.

Several biochemical investigations have indicated the presence of opiate presynaptic receptors on axons and nerve terminals (*2*). Light microscopic autoradiographic studies have demonstrated opiate receptors in association with the rat vagus nerve fibers and with other small diameter nerve fibers (*3*, *4*). It was suggested that some of these vagal receptors may be undergoing axonal transport or axoplasmic flow (*4*). In vitro labeling autoradiography, a highly sensitive and quantitative measure of receptors (*5*), has proved adequate for measuring opiate receptors in the vagus. By using this technique, we detected opiate receptors in the nodose ganglion and receptor buildup on the proximal side of a ligature of the vagus nerve.

Surgical exposure of the nodose ganglion and vagus nerve in the neck region of the rat was performed as described in

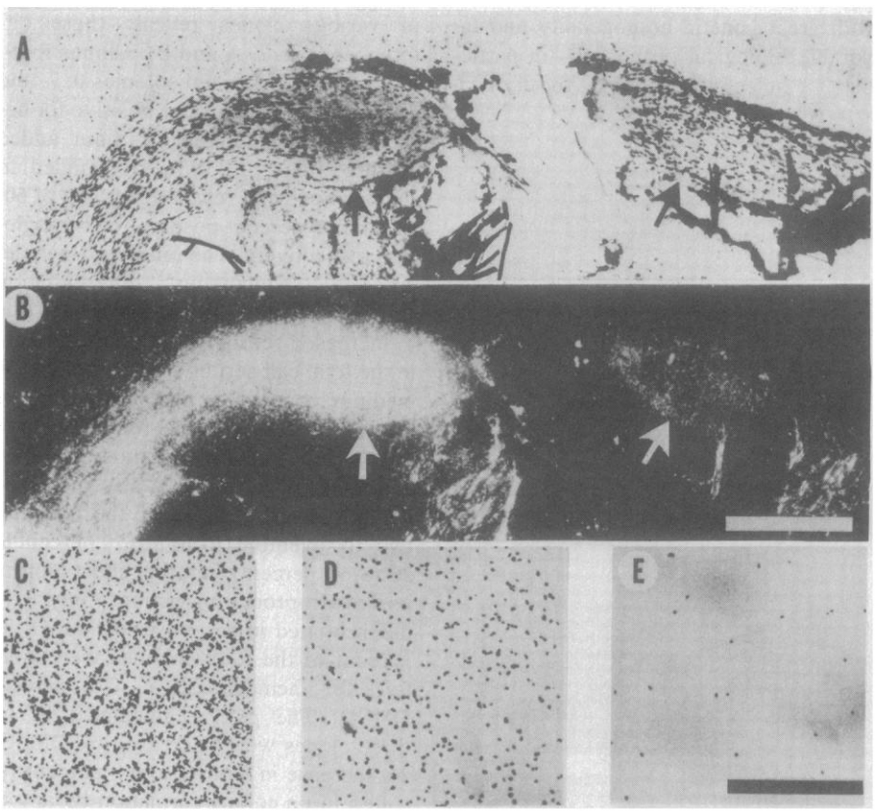

Fig. 1. (A) Light micrograph showing a section along a ligated nerve. The nerve was removed from the neck and embedded in a brain paste to maintain a good consistency for sectioning. The ligation was placed slightly to the right of center where there is an actual separation of proximal (left) and distal (right) sides. Arrows point to the nerve sheath or edge of nerve on either side of where the tie was made. Dark streaks to right of both arrows are fragments of the silk ligature. The autoradiographic grains marking receptors cannot be seen at this magnification with brightfield microscopy. (B) A darkfield of the same section as in (A). The grains appear as white dots and the tissue cannot be seen. The grain density is so high on the proximal side that the individual grains cannot be distinguished. Scale bar, 500 μm. (C) A high-power brightfield taken directly above the left arrow in (B) showing the grain density on the proximal side. (D) Grain density on the distal side directly above the right arrow in (B). (E) Grain density from an adjacent section that was coincubated with naloxone to produce a blank. The photograph shows the same part of the nerve as that in (D). Scale bar, 50 μm. Autoradiograms were exposed for 3 months. The receptors were labeled with [^{3}H]DAMA.

0036-8075/80/1003-0076$00.50/0