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11. In some experiments the β-blocker propranolol was used instead of sotalol. However, prevention of the β-receptor decrease by propranolol could not be demonstrated, because it was difficult to remove residual propranolol from the slices.

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Hemoglobin Switching: A Cellular Model

Abstract. *Regulation of hemoglobin synthesis depends in part on the population of cells available for erythroid differentiation. Mouse erythroleukemia cells were cloned, and the clones were induced with dimethyl sulfoxide to test the relative induction of β minor and β major synthesis. Cells of line 745 produced approximately 35 percent β minor after induction, and 39 clones of line 745 produced from 23 to 61 percent β minor. Further subcloning of the clone that produced 61 percent β minor led to three subclones, all of which produced more than 90 percent β minor. Thus one kind of hemoglobin regulation occurs at the cellular level.*

Regulation of hemoglobin synthesis may occur at the cellular or the subcellular level. At the cellular level, modulation of globin expression results from growth and differentiation of cell populations with specific programs for globin synthesis. At the subcellular level, modulation depends on alterations in transcription, processing, or translation of globin genes. The relative contribution of cellular versus subcellular regulation in the types of hemoglobins produced is not established. We now present a murine model of erythropoiesis designed to determine the potential contribution of cellular selection in hemoglobin regulation. Mice of strain DBA/2 produce a major ($\alpha_2\beta_2$-major) and a minor ($\alpha_2\beta_2$-minor) hemoglobin. In utero the proportion of minor may exceed major, but in the adult the ratio of major to minor is approximately 80 to 20 (*1*). In mice, the shift from minor to major qualitatively resembles the switch from fetal to adult hemoglobin in humans. Mouse erythroleukemia cells, developed from DBA/2 mice infected with Friend leukemia viruses, provide a model for the switch. The cells grow in suspension as proerythroblasts, undergo erythroid differentiation when cultured with inducing agents, and produce mouse hemoglobin (*2*). The relative proportions of the major and minor hemoglobins depend on the inducing agent, as well as on the cell line. For example, induction of line 745 with dimethyl sulfoxide leads to synthesis of approximately 30 percent β minor while induction of the same line with hemin leads to 100 percent β minor.

Cytoplasmic messenger RNA (mRNA) which was obtained from induced erythroleukemia cells directed, in a wheat germ cell-free system, the synthesis of globin chains in a pattern similar to that seen in the induced intact cells (*3*). Thus inducing agents might alter globin gene transcription in erythroleukemia cells. However, these agents might also induce selective growth and differentiation of cells within the mass culture preprogrammed to produce a specific pattern of globin chains. To test this possibility, we cloned line 745 cells without inducing agents, and tested the pattern of β chain synthesis after induction of the clones with dimethyl sulfoxide. Cellular heterogeneity found in the induction patterns suggests that one form of hemoglobin regulation occurs at the cellular level.

Erythroleukemia cell line 745 was grown in Dulbecco's modified Eagle's medium in 10 percent fetal calf serum in an atmosphere of 10 percent CO_2 and passaged twice weekly (*4*). The line was in continuous culture for 2 years. For cloning, cells in the log phase of growth were suspended in medium at one cell per 2 ml, and 0.2 ml was placed in each well of Falcon Microtest II dishes. After 8 to 10 days, 10 percent of the wells had a visible clone in one area of the bottom of the well. Each clone was replated in 2 ml of medium in 35-mm Falcon dishes. When the cell density reached 1×10^6 to 3×10^6 cell/ml, the clones were replated at 2.5×10^4 cell/ml in 10 ml of medium (in 40-ml Falcon flasks) and passaged twice at that density to maintain logarithmic growth.

The cloned cells were then plated at 5×10^4 cell/ml in 10 ml of the medium described above, to which 2 percent (280 m*M*) dimethyl sulfoxide was added. After 4 days, the cells were centrifuged, and resuspended in 2 ml of medium lacking leucine, to which 25 μCi of ^{3}H-labeled leucine ([3,4,5-^{3}H]leucine, 100 Ci/mmole; New England Nuclear) was added. After incubation at 37°C for 4 hours, the cells were harvested by centrifugation and washed three times with Hanks balanced salts with 1 percent bovine serum albumin. The cell pellets were lysed by the addition of 50 or 100 μl of 1 m*M* Na_2HPO_4 (*p*H 7.4) and incubation at 37°C for 15 minutes. Stroma and nuclei were removed by the addition of 25 μl of a mixture of carbon tetrachloride and toluene (1 : 1), vigorous vortexing, and centrifugation for 4 minutes at 10,000*g* (Brinkmann Microfuge 3200). Lysates were stored in liquid nitrogen.

Globin chains were analyzed by polyacrylamide gel electrophoresis (containing acid, urea, and 2 percent Triton X-100) (*5*) (Fig. 1). The β major, β minor, and α chains of mouse globin separate from each other and from nonglobin proteins. With this assay, globin synthesis was not detected in uninduced cells of line 745, but was seen in the electrophoresis of newly synthesized radioactive proteins from line 745 that had undergone induction with 2 percent dimethyl sulfoxide for 4 days (Fig. 1B). The gels were processed by fluorography (*6*), and the relative rates of globin chain synthesis were determined by integration of the areas under the peaks detected by densitometry (Fig. 2A). The β minor represented 32 percent of the total β chain synthesis. Line 745 was induced and globin synthesis was analyzed in 12 separate experiments (Fig. 3). The mean percentage of β minor was 35, with a range from 32 to 41, and 1 standard deviation of 3 percent.

In the absence of inducer, 39 clones of line 745 were obtained and then induced with 2 percent dimethyl sulfoxide for 4 days; globin synthesis was then evaluated (Fig. 3). The mean β minor was 32 percent, similar to the mean in the 12 studies of line 745. However, the range

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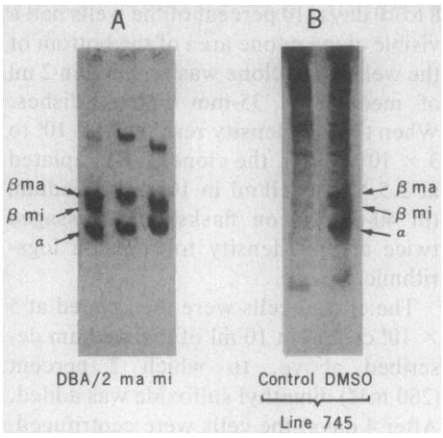

Fig. 1. Electrophoretic separation of mouse globin chains. Whole lysates were subjected to electrophoresis on acid, urea, and 2 percent Triton X-100 polyacrylamide slab gels (*5*). (A) Gel stained with Coomassie blue. (Left) DBA/2 adult mouse hemoglobin, (middle) major hemoglobin, and (right) minor hemoglobin. The major and minor hemoglobins were purified by chromatography of adult mouse hemolyzate on diethylaminoethyl-cellulose, and elution with glycine-KCN (*p*H 7.8) with a NaCl gradient (*10*). (B) Fluorogram of [^{3}H]leucine-labeled proteins from line 745 erythroleukemia cells on day 4 of culture. Slab gels were treated with dimethyl sulfoxide (*DMSO*) and 2,5-diphenyloxasole, dried, and exposed to preflashed Kodak XR2 X-Omat film (*6*). (Left) Uninduced cells, showing no globin synthesis; (right) dimethyl sulfoxide-induced cells, showing globin synthesis, as well as separation of globin from nonglobin proteins.

was from 23 to 61 percent, and one standard deviation was 6 percent. The difference in the dispersion of the percentage of β minor in line 745 and its clones was statistically significant ($P < .01$) (*7*). These results suggest that cellular diversity contributed to the wide range of β minor synthesis.

Clone 25, which produced 61 percent β minor, was investigated further (Fig. 2B). Six separate experiments over a 6-month period led to a mean of 57 percent for β minor synthesis (range, 53 to 62 percent). Soon after it had been obtained, we subcloned clone 25. Ten subclones were induced with dimethyl sulfoxide and produced a mean of 56 percent β minor (range, 52 to 67 percent). These subclones thus resembled the parent, indicating cellular homogeneity. One subclone, called 25-6, was cultured for 4 months, stored in liquid nitrogen for 2 months, cultured for an additional 2 months, and then subcloned. Induction of ten subclones of 25-6 with dimethyl sulfoxide led to the synthesis of a mean β minor of 73 percent (range, 52 to 97 percent; standard deviation, 16). Three of the subclones produced more than 90 percent β minor (Figs. 2C and 3). Thus culture or freezing and thawing (or both) of clone 25-6 led to heterogeneity in the cell population.

In these experiments the type of β chain produced as a result of induction with dimethyl sulfoxide depended on the specific clone of cells being tested. We began with line 745, which produced 30 percent β minor and 70 percent β major when tested by induction with dimethyl sulfoxide. Progressive cloning in the absence of inducer led to the selection of cells with a diverse pattern of dimethyl sulfoxide-induced β chain synthesis, including several in which β minor was now the "major" β chain.

Thus, in the murine erythroleukemia model, single cells were isolated with apparently diverse hemoglobin programs, which were expressed by culture with an inducing agent. Since the uninduced erythroleukemia cells are proerythroblasts (*2*), commitment to a specific type of globin synthesis may occur at the level of the proerythroblast when the erythroid cells are still dividing and morphologic and biochemical markers for erythroid differentiation have not yet appeared. Studies of human or sheep erythroid colonies grown in vitro suggest that the type of hemoglobin produced

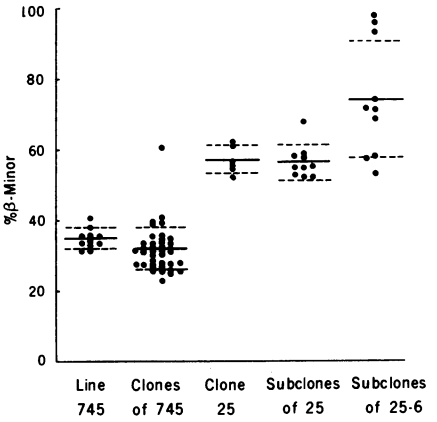

Fig. 3. Percentage of β minor synthesis in erythroleukemia cells. Each line or clone was obtained and carried without inducing agents. The cells were cultured for 4 days in 2 percent dimethyl sulfoxide, and labeled with [^{3}H]leucine for 4 hours. Newly synthesized proteins were subjected to electrophoresis on Triton gels, fluorographed, and scanned with a densitometer [percent β minor = area under β minor/(β major + β minor) × 100]. Statistical analyses were done with the Siegel-Tukey test, a nonparametric, sums-of-rank procedure for relative spread in unpaired samples (*7*). For line 745, 12 independent experiments were analyzed. For clones of 745, 39 clones were obtained from line 745 and analyzed in groups of ten. For clone 25, six independent experiments were performed over a 6-month interval. For subclones of clone 25, ten subclones were obtained from clone 25, 2 months after clone 25 had been derived. For subclones of subclone 25-6, ten subclones were obtained from subclone 25-6, which had been cultured for 4 months, stored in liquid nitrogen for 2 months, cultured for 2 months, and then subcloned. (—) Mean; (----) 1 standard deviation.

may be determined by commitment of progenitors which are even earlier in development than proerythroblasts (*8*). This cellular commitment does not preclude the possibility of additional regulation of hemoglobin production by effects on transcription of globin genes, processing of globin RNA, or translation of globin mRNA. However, our findings suggest that selective growth and differentiation of erythroid precursor cells with specific hemoglobin programs can influence the type of hemoglobin ultimately produced in vitro and probably in vivo (*9*). Cultures of cells committed to a specific globin program, such as our clones that produce only β minor, will be useful for further investigation of the molecular basis of hemoglobin regulation.

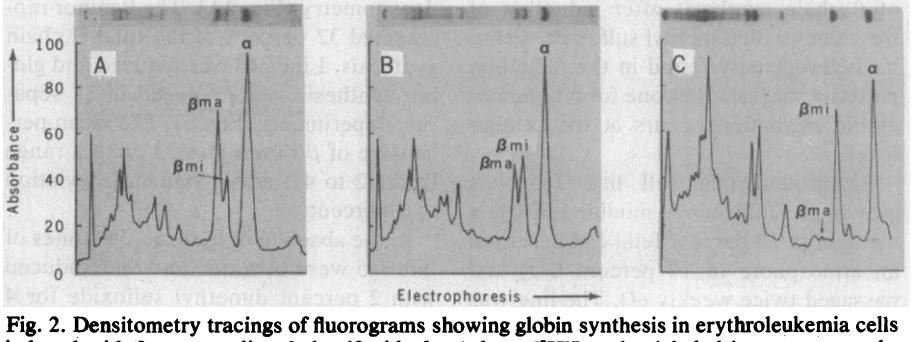

Fig. 2. Densitometry tracings of fluorograms showing globin synthesis in erythroleukemia cells induced with 2 percent dimethyl sulfoxide for 4 days. [^{3}H]Leucine-labeled lysates were subjected to electrophoresis on Triton gels. The inset in each figure shows the pattern of the fluorogram. The fluorograms were scanned with an E-C densitometer. (A) Line 745; β minor, 32 percent; (B) clone 25; β minor, 61 percent; (C) subclone 25-66; β minor, 97 percent.

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Postsynaptic Transmission Block Can Cause Terminal Sprouting of a Motor Nerve

Abstract. *Sprouting of mouse soleus motor nerve terminals can be evoked by daily intramuscular injections of purified α-bungarotoxin. This finding supports the hypothesis that an important stimulus to terminal sprouting in partial denervation and presynaptic nerve blockade is a product of inactive muscle fibers.*

The factor or factors responsible for controlling new growth from adult motor nerve terminals are still unknown. That terminal sprouting can be evoked by presynaptic blockade of transmission, by either botulinum toxin (*1*) or tetrodotoxin (*2*), has led to the suggestion that the sprouting signal arises from inactivated muscle fibers, which are, of course, also present in partially denervated muscles. However, it is also claimed that blockade of axoplasmic transport without interference in neuromuscular transmission leads to sprouting (*3*). Presynaptic blockers were thus suggested to cause sprouting by preventing the release of a neuronally transported antisprouting agent (*3*). That directly activating the paralyzed muscles inhibits the expected sprouting produced by botulinum toxin paralysis (*4*) militates against this hypothesis. We now present further evidence to support an important role for inactive muscle in producing terminal sprouting. By giving repeated injections of a postsynaptic blocker—purified α-bungarotoxin (α-bgt) (a highly specific noncompetitive blocker of the acetylcholine receptor that is unlikely to have any direct effects on the nerve)—we have, in contrast to previous reports (*5*, *6*), produced significant terminal sprouting.

The experiments were carried out on the soleus muscles of female Olac mice (20 to 35 g). The technique was essentially as described elsewhere (*7*). Several tests allowed us to be sure that we were dealing with α-bgt (*8*). It is important that pure toxin be used; commercially available "purified" α-bgt can cause degeneration of motor nerve terminals and a long-lasting paralysis after just one injection. The toxin we used causes a temporary paralysis with no degeneration, which is why repeated injections were required.

The toxin was dissolved in mammalian Ringer solution to which had been added 2 mg of bovine serum albumin (BSA) (fraction V, BDH biochemicals) per milliliter of solution to give a final toxin concentration of 1 mg/ml. The BSA was necessary to prevent the adsorption of the toxin to glass. The mice were sedated with 3.5 percent chloral hydrate (0.1 ml per 10 g of body weight) injected intraperitoneally before each injection of toxin, in an attempt to improve the localization of the toxin. The toxin was injected with a 10-μl Hamilton syringe directly into the back of the leg just above the Achilles tendon. The mice received 1 μg daily or 0.5 μg twice daily. Control mice were treated identically, but received only BSA in Ringer solution. Botulinum toxin (crude type A) was injected in the same way; one dose of 0.03 μg in 3 μl produced a profound, local, long-lasting paralysis. The physiologic experiment took place between 4 and 8 days after starting the injections, times at which sprouting would be expected to be well under way (*2*). The mice were anesthetized (0.2 ml of 3.5 percent chloral hydrate per 10 g of body weight), and the right soleus muscle and nerve were dissected free. The muscle was studied in vitro; the isometric nerve and directly evoked twitch tensions and the 50-Hz tetanic tensions were measured. The muscle's acetylcholine sensitivity was assessed by comparing its contracture in 5×10^{-4} g per milliliter of acetylcholine perchlorate with the maximum 50-Hz direct tetanic tension. Finally, each muscle was pinned out on a Sylgard-bottomed dish and stained for 6 hours in zinc iodide and osmium tetroxide. The muscles were teased apart with fine forceps, dehydrated in ethanol, cleared in xylene, and mounted in Permount. The observer examined all slides without knowing to which group it belonged and noted for each end plate examined its length in the long axis of the muscle and the corresponding length of any sprouting of that end plate. Fifty or more randomly chosen end plates were examined in each muscle. The amount of sprouting was expressed as the percentage of the end plates examined that had terminal sprouts. Normal solei have, on average,

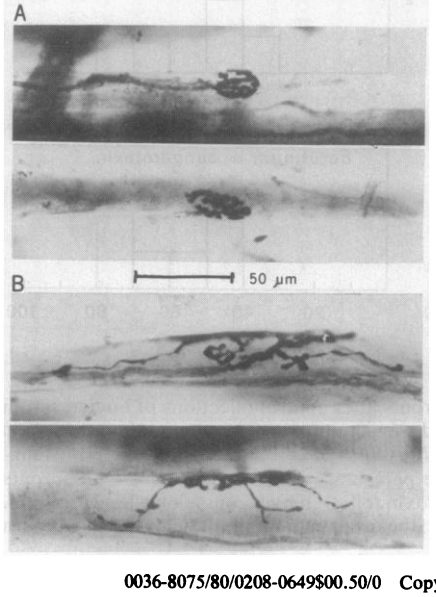

Fig. 1. End plates, stained with zinc iodide and osmium tetroxide, in mouse soleus muscles. (A) Two end plates of normal appearance from a muscle treated for 6 days with a daily injection of 1 μl of Ringer solution containing 2 μg of bovine serum albumin. (B) Two end plates with fine terminal sprouts from a muscle treated for 7 days with a single daily injection of 1 μl of Ringer solution containing 1 μg of purified bungarotoxin and 2 μg of bovine serum albumin. The virtue of the zinc iodide-osmium technique is that it stains the unmyelinated portions of the nerves in their entirety.

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