ERYTHROPOIETIC CELL CULTURES FROM CHICK EMBRYOS

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ABSTRACT

Erythropoietic cell cultures from very early chick blastoderms survive for several days They show four to seven doublings of the erythroid cells and the appropriate morphological changes from proerythroblasts to mature erythrocytes Cell cycle times are the same as *in ovo* for the first day of culture, but slow down thereafter The hemoglobins of both the primitive and the definitive red cell series are produced. 5-Bromodeoxyuridine added to the cultures inhibits differentiation and hemoglobin synthesis, though not cell division, but quite soon the cells cease being sensitive The effect of the drug can be reversed by the addition of thymidine.

Red blood cell formation, erythropoiesis, in chick embryos has been studied for many years in vivo (2, 4, 5, 14, 34, 35) and more recently in vitro through short-term organ culture of the blastoderm (8, 10, 15, 17–21, 23, 27, 29, 30, 35). Erythroid precursor cells arise in vivo in the mesenchyme at about 24 hr of incubation or even earlier, presumably by a process of induction by endodermal cells, as shown by a classical transfilter experiment by Miura and Wilt (17). Early erythroid precursor cells appear in clusters and are morphologically identifiable in the light- and electron microscopes (2, 9, 24, 25, 28, 33). Hemoglobin begins to be detectable at 36 hr of incubation; a circulatory system forms at 48–60 hr. The early embryonic hemoglobins seem to be the product of dividing and maturing cells of the so-called "primitive" red cell series, a cohort of cells (4) appearing as a group in the course of a day or so. Thus, the primitive cells tend to be at the same maturation stage at the same time. They have all stopped dividing by day 5; thereafter, they continue to synthesize hemoglobin at a decreasing rate, circulate for a few days as mature red cells, carry out their oxygen transporting function in the embryonic circulation, and then disappear (4, 5, 14, 25, 34, 35); few are formed after day 13.

Their place in the circulation is taken after day 7 by the second and permanent red cell series (4, 5, 14, 25, 34, 35)—the morphologically distinct "definitive" red cells carrying the adult hemoglobin types (4, 35). In the chick embryo this is a self-renewing population, sequestered in the yolk sac tissue and later in the marrow, but separate from the circulation. The sequestered population contains the stem cells and the most immature stages of the definitive red cells. The cellular origin of the precursors of the definitive red cell line is quite unknown; their origin and the timing of the appearance of this second red cell line are prime targets of present-day research. Organ culture experiments (27, 35) covering development to day 2.5, as well as organ culture (8) and *cell culture* work in our own laboratory, covering also the appearance of the definitive red cell series and its hemoglobins, show promise that a biochemical approach to this system is possible.

There is a close and obvious relationship be-

tween embryonic erythropoiesis in the chick and that in man, the mouse, and the tadpole (12), so that the problem of the determination which leads to the appearance of the different red cell population is of general interest. It is probably best studied in cell culture.

The purpose of the present communication is to describe an erythropoietic *cell culture* from very early chick embryos which survives for several days and which shows four to seven doublings of the erythroid cells and the appropriate morphological changes from proerythroblasts to mature erythrocytes.

MATERIALS AND METHODS

Fertilized eggs of the White Leghorn breed were obtained from the Spafas Co., Norwich, Conn. Incubation was carried out for either 24 or 34 hr, as indicated for each experiment. Media, sera, and antibiotics were obtained from the Grand Island Biological Co., Grand Island, N. Y. For most of the experiments, the culture medium consisted of Nutrient Mixture F12 with glutamine (7 parts), a particular batch (e.g., A8072D) of heat-inactivated fetal calf serum (1 part), and also penicillin, streptomycin and fungizone (100 units/ml, 100 μg/ml, and 0.25 μg/ml, respectively.) Suitable batches of serum were selected by testing them for their ability to promote cell proliferation at the usual rate. Either heat inactivation was done at 56°C for 30 min, or the serum was purchased as "heat inactivated"

The blastoderm, still attached to the vitelline membrane, is cut out and removed from the main body of yolk. Gentle swirling in Howard Ringer's solution can be used to remove much of the attached yolk from the blastoderm. The blastoderm is detached from the vitelline membrane, and several blastoderms are transferred to a sterile tissue culture dish (Falcon Plastics Division of B-D Laboratories, Inc., Los Angeles, Calif, 35 × 10 mm) containing 1 ml of medium. They are cut up with dissecting scissors, pumped up and down in a sterile 9 inch Pasteur pipette until a fine suspension is obtained (about seven times), then transferred to a sterile 5 ml Erlenmeyer flask with a screw-cap top. The culture is incubated at 39 5°C on a gyratory table which swirls the culture at a slow but even speed (circular motion, approximately 60 rpm, radius of gyration = 0.6 cm) This motion keeps erythroid cells in suspension, but allows most of the other cell types to attach to the glass. Some yolk globules also remain in suspension.

The numbers of erythroid cells in the cultures were determined by preparing slides from the cell suspension, diluted 1:10 in Hanks' containing 1% bovine serum albumin, in the Cytocentrifuge (Shandon-Elliot, Sewickley, Pa., 10 min at 600–700 rpm). Cells were stained with benzidine for hemoglobin and counter-stained with May-Grünwald-Giemsa stains. By this means erythroid cell numbers could not be obtained by direct counting in a hemocytometer, since some yolk globules and a few unattached non-erythroid cells were still present. Therefore, a known concentration of mature sheep red cells was included when the above-mentioned slides were made. Mature sheep red cells are easily recognized, since they are much smaller and are not nucleated, whereas even mature chick red cells of both the primitive and the definitive series retain their nuclei. A differential count of chick and sheep cells gave the absolute number of chick red cells. In most cases, at least 200 sheep cells and 50–400 chick cells were counted for each culture and each time point. The numbers so obtained are approximate, and therefore each experiment was done at least in duplicate. There was also considerable variability from week to week. On the first and second days of culture, some clumps of erythroid cells were usually present, but on later days only single cells were seen.

Extraction and estimation of hemoglobin from cells were carried out as described by Hagopian and Ingram (8), and examination of such hemoglobin extracts was as cyanmethemoglobin by acrylamide gel electrophoresis (16). Radioautography was carried out, as described (8), on cells labeled with thy-

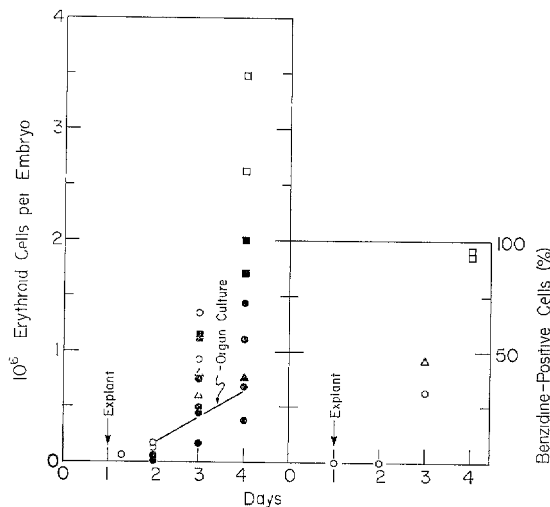


FIGURE 1 Yield of erythroid cells and appearance of hemoglobin-containing cells in suspension cultures from 24-hr blastoderms (definitive streak or head process stage). Different symbols refer to different experiments: L33 (●), L30 (■), L29C (□), H79 (○), L62 (△), L64 (▲). The line marked "organ culture" refers to the cell numbers obtained in previous experiments by culturing intact blastoderms (Reynolds and Ingram [23]).

midine-^{3}H (New England Nuclear Corp., 6.7 Ci/mmole).

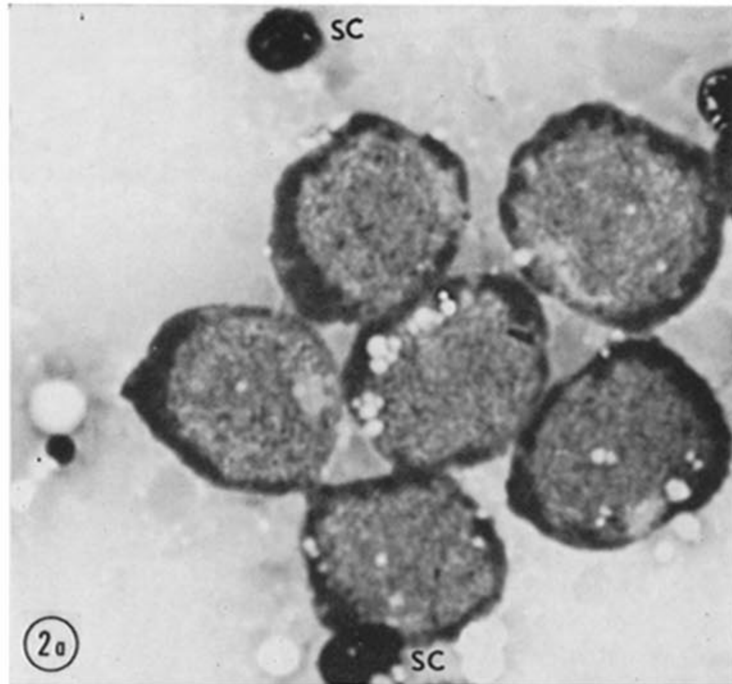
RESULTS

Our initial aim was to establish a technique which would produce reasonably pure cell cultures consisting of erythroid cells, which showed a significant number of cell doublings and which had the morphological and hemoglobin-producing characteristics of normal embryonic red cells of both the primitive and the definitive cell lines Such erythroid cell cultures have previously been difficult to obtain. We wanted to keep the conditions of culture as close as possible to those which obtain in the early embryo, since our object is the study of red cell development rather than the production of a cell line in culture. Therefore, we used whole early embryos, disaggregated mechanically. Secondly, since red cells are known to lose their adhesiveness as they differentiate from proerythroblasts to the mature erythrocyte, and since the late maturation stages are characteristically in suspension all the time, we used a gentle gyratory motion for our culture flasks. This resulted in our obtaining suspensions of differentiating red cells, plus some of the original yolk particles, while unwanted cells of fibroblastic or epithelial type, etc., adhered to the glass vessel and thus were largely removed from our suspensions.

Red cell production was quite variable, as can be seen in Fig. 1, where numbers from individual flasks in different experiments are given, rather than average values. After day 4, there is usually, but not always, a sharp drop in cell numbers. However, there is continued maturation and further hemoglobin production in those cells that survive. Erythroid cell numbers at the time of explantation are difficult to determine, because at that time some of the erythroid cells are still in clumps (blood islands?) which later disperse. Between day 2 and day 4, there are clearly between two and five doublings This estimate does not include the events of the first day of culture, which, according to measurements of cell cycle time (see later), can be expected to include at least another two doublings Altogether, the cultures show four to seven doublings of erythroid cells during 72 hr of culture and therefore doubling times in the range of 10–18 hr, a reasonable range, and comparable to the in vivo values at that time (34) and to those obtained by Hell (10) and by us in organ culture (8). For comparison, the line in Fig. 1 is the yield of red cells obtained from 24-hr blastoderms in organ culture (23). Although the cell numbers are comparable at explantation (by definition) and on day 2 (by measurement), thereafter the suspension cell cultures do much better. Suspension cell cultures prepared from 31- or 34-hr blastoderms (head fold stage and up to three somites) show a similar pattern of doubling behavior, with slightly higher cell numbers, also followed by a decline. However, the 24 hr blastoderm culture does rather poorly when fewer than four blastoderms are placed in culture per milliliter. The older blastoderms, as expected, can be successfully cultured at two blastoderms per milliliter.

During their 3 days in culture, the cells go through the expected morphologically recognizable differentiation stages (see insert of Fig. 1 and photos in Fig. 2). Morphological development is normal although perhaps just a little slower than in vivo, sometimes vacuolation of cells is seen and sometimes the final erythrocytes are hypochromic. Hemoglobin was extracted from the cells of culture L33 (Fig. 1) on day 4 and examined by gel electrophoresis (Fig. 3). Clearly the cells contain all four hemoglobins characteristic of the system. The hemoglobins P and E of the primitive cell line are present and so are hemoglobins A and D of the definitive cell line, found in the late embryo and the adult chicken. The proportions of hemoglobin A and D relative to P and E are low, indicating more primitive cells or less hemoglobin A and D per cell. The immature precursors of the definitive cell line, carrying hemoglobins A and D, are apparently sequestered in the embryo (4). Therefore, these hemoglobins are not found in circulating blood of intact embryos in the proportions of Fig. 3 until day 7. The early appearance of hemoglobins A and D in hemoglobin extract from our culture could be due to an early appearance of definitive cells, or to the unexpected turning on of late hemo-

FIGURE 2 Photographs of cells from suspension cultures. Cells stained with benzidine and May-Grunwald-Giemsa. (*a*) Day 3; basophilic erythroblasts (*BE*); (*b*) day 3, proerythroblasts (*PE*), basophilic erythroblasts (*BE*), polychromatophilic erythroblasts (*P*); (*c*) day 5, almost mature erythrocytes, presumably of the primitive series. The small densely stained cells (*SC*) are sheep cells added for counting. Fig. 2 *a*, × 2000; Fig. 2 *b*, × 800; Fig. 2 *c*, × 800.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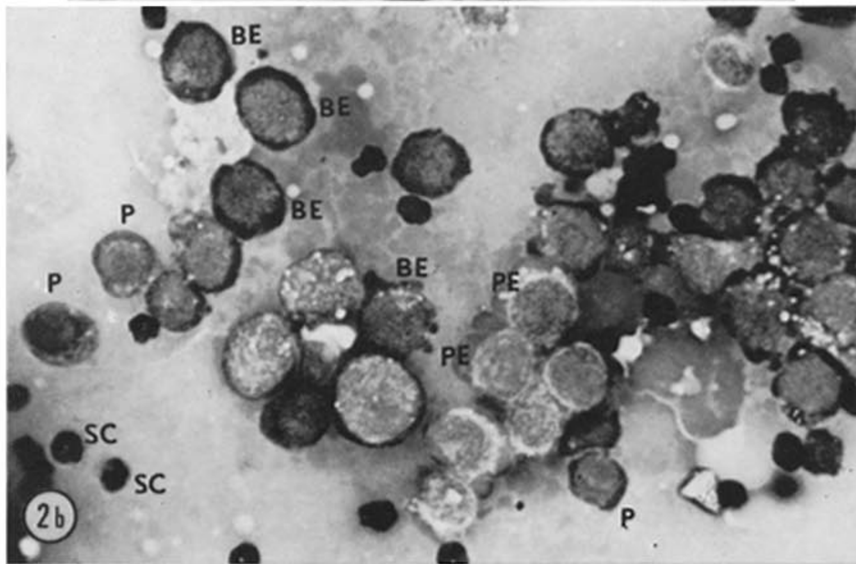
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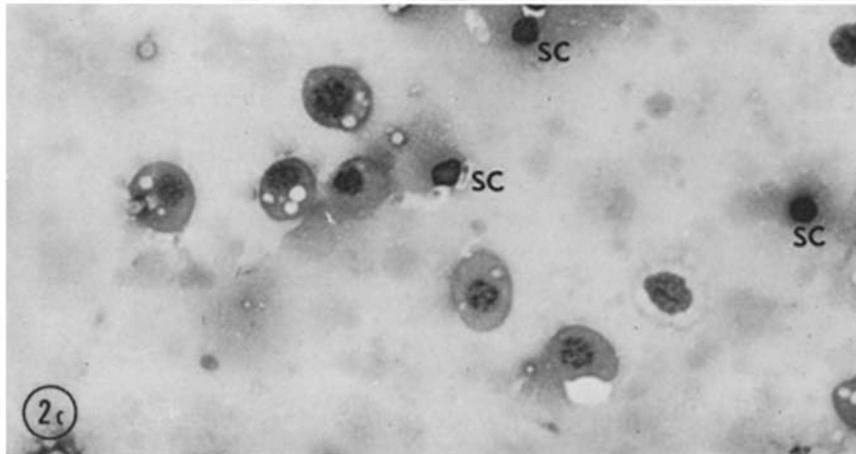
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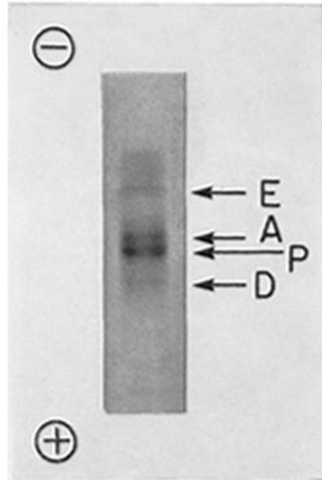

FIGURE 3 Acrylamide gel electrophoresis of the hemoglobins extracted from a culture (L33) on day 4. *E* and *P* = hemoglobins E and P of the primitive red cell series; *A* and *D* = hemoglobins A and D of the definitive red cell series. Benzidine stain.

globin genes in primitive cells, or to selective decay and early disappearance of primitive cells in culture. It should be added that both primitive and definitive cells can be seen in some of our cell cultures on day 4 and later, although morphological characterization of the different red cell series is much more difficult in cultures than *in ovo*

Cell cycle times during culture were determined by the Colcemid collecting function of Puck (22). At 0.04 μg/ml of Colcemid, mitoses were accumulated during 6 hr, without a lag, at the same rate as at 0.2 μg/ml. The lower concentration was used and is illustrated in Fig. 4. Samples were taken every 1.5 hr for 6 hr, and about 800 cells were counted to determine the proportion of mitotic cells at each time point. Since there seemed to be a slight decrease in the rate of accumulation after 3 hr in Colcemid, perhaps due to slow escape from arrest, the values of the cell cycle time and for mitosis given in Fig. 5 are derived from the first 3 hr of treatment. They might represent a maximum value if there is escape from arrest. Only erythroid cells were used for these observations, using particularly the criteria of basophilia, nuclear staining, and the absence or presence of benzidine staining for interphase cells, and using basophilia and the absence or presence of benzidine staining for cells in mitoses. The results given in Fig. 5 for the earlier ages compare with those obtained *in ovo* by Weintraub et al. (34) and in organ culture by Hell (10)

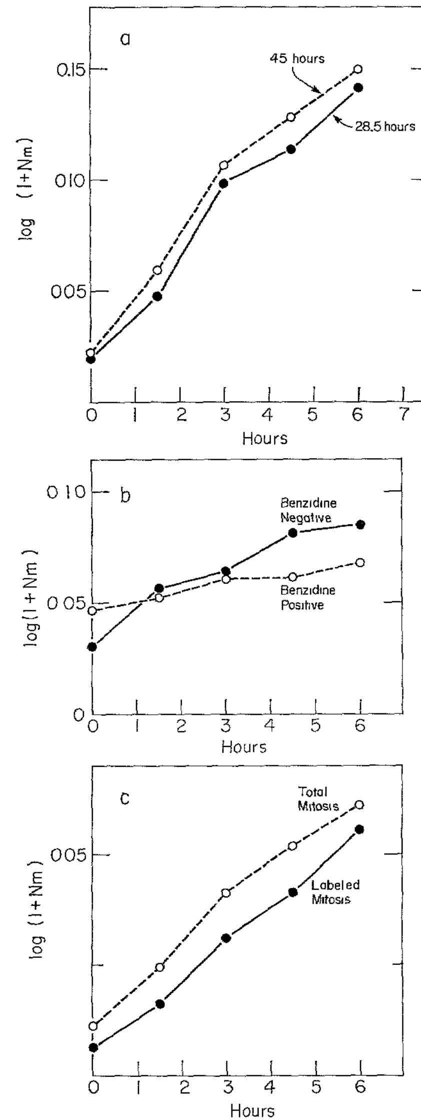

FIGURE 4 Accumulation of cells caught in mitosis in Colcemid (0.04 μg/ml) as displayed by the "collecting function" of Puck (22). N_m is the fraction of cells in mitosis at a particular time after addition of Colcemid. The slope of this function gives the average cell cycle time of the culture. Results of duplicate experiments are given. Cultures illustrated in this figure were prepared from 24-hr blastoderms and exposed to Colcemid at a total age of (*a*) 28.5 and 45 hr or (*b*) 69 hr. Other cultures (*c*) were derived from 36-hr blastoderms and exposed to Colcemid and thymidine-^{3}H when the cells had reached a total age of 79 hr. Radioautography was performed on slides from culture (*c*). The results from these and other experiments are displayed in Fig. 5.

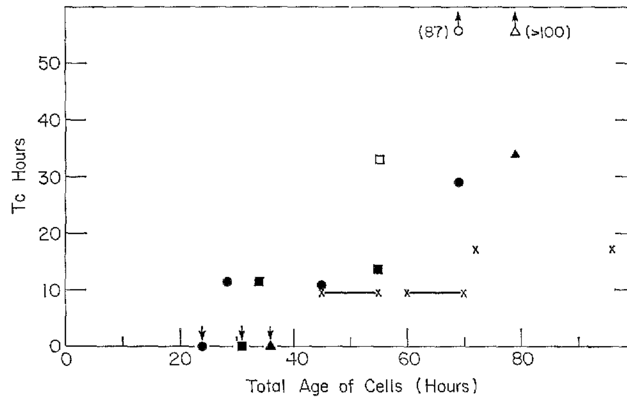

FIGURE 5 Cell cycle times (*Tc*) of cultures at various ages. Age of the embryo at time of explantation (arrow) of a particular cultures is indicated by the appropriate symbol (●, ■, ▲) on the abscissa The filled symbols refer to benzidine-negative cells; open symbols refer to benzidine-positive cells. Measurements of the time spent in mitosis (Tm) gave values of 0.45, 0.6, and 0.75 hr during cell ages of 29–45 hr. The in vivo values of Weintraub et al. (34) are also shown (×); the line connecting some of these points indicates that intermediate values are similar.

and by Hagopian and Ingram (8) but are 1–2 hr slower. Whereas Weintraub et al. measured cell cycle times for the whole circulating population, we measured separate values for benzidine-negative and benzidine-positive (older) cells.

In order to ascertain that all erythroid cells were in cycle when these Colcemid experiments were done, thymidine-^{3}H (1.6 μCi/ml) was added to a culture, at total cell age of 79 hr, at the same time as the Colcemid (Fig. 4 *c*). Slides were processed for radioautography as described. The benzidine-negative cells, i e. basophilic erythroblasts, which are still numerous, are also still cycling, albeit more slowly, at 34 hr per cycle. As Fig. 4 *c* shows, the rate of accumulation of labeled mitoses is the same as the rate for total mitoses, with a delay of only 1 hr This is at least an indication that, even at this late stage in culture, all cells of that morphological type are in cycle. Therefore, it is likely that at earlier times the same healthy condition exists

The effect of 5-bromodeoxyuridine (BUdR) on our suspension cultures was investigated after the report by Miura and Wilt (19) that the drug will prevent the appearance of erythropoietic foci in organ cultures of reaggregated early blastoderms. The concentration of BUdR used by these authors (40 μg/ml and more) was lethal to our cell cultures, most of our experiments were done at 4 μg/ml, and more recently at 8 μg/ml. Suspension cultures from 24-hr blastoderms (a mixture of definitive streak, head process, and head fold stages responded to 8 μg/ml of BUdR by a drastically reduced rate of differentiation to morphologically mature, hemoglobin-containing cells (Table I), with little effect on cell numbers. It must also be recalled that BUdR-treated cells are reported as more flattened and more adhesive (1, 3). Such an effect would lower the number of cells harvested by us in our suspensions. Those cells which did differentiate stained much less strongly for hemoglobin, i.e., hemoglobin production appeared to have decreased.

Adding thymidine abolished the BUdR effect on the maturation and hemoglobin production of the cells, as had been reported by Wilt (35), but quite high concentrations were needed: in the experiments shown, the concentration was 100 μg thymidine/ml. This concentration of thymidine alone had no effect on the rate of morphological differentiation (Table I) or on cell numbers. The simultaneous addition of thymidine and BUdR at the time of explantation almost completely counteracted the BUdR (Table I), but even addition of thymidine 5 or 10 hr after BUdR led to complete reversal (Table I). However, after 21 hr, only partial reversal was obtained by day 4 or 5. These results agree approximately with the findings in organ culture of Miura and Wilt (19).

Probably the reason why BUdR at the time of explantation did not completely suppress differentiation is the presence in the proerythroid cell population of a certain number of cells which are older and therefore no longer sensitive to BUdR, as suggested by Miura and Wilt (19). Therefore,

TABLE I

The Effect of BUdR on the Differentiation of Erythroid Cells in Cultures Derived from 24-hr Blastoderms and Its Reversal by Thymidine

Time of addition (hr after start of culture)		Day 3		Day 4		Day 5	
BUdR*	TdR‡	× 10⁶ cells/ml	% of benzidine-positive cells	× 10⁶ cells/ml	% of benzidine-positive cells	× 10⁶ cells/ml	% of benzidine-positive cells
None	None	2.6	47			3.4	100
None	0	3.2	64			4.0	100
0	None	1.3	18	3.1	19	2.2	32
0	0	1.5	23			3.4	98
0	5			5.1	85	3.6	99
0	10			3.2	80	3.3	96
0	21			4.5	56	5.0	78

* 5-Bromodeoxyuridine, 8 μg/ml; cultures have four dissociated blastoderms/ml; duplicate experiments.
‡ Thymidine, 100 μg/ml.

TABLE II

The Effect of BUdR Additions at Various Times on the Differentiation of Erythroid Cells in Cultures Derived from 20-hr Blastoderms

Time of BUdR addition* (hr after start of culture)	Day 3		Day 4	
	× 10⁶ cells/ml	% of benzidine-positive cells	× 10⁶ cells/ml	% of benzidine-positive cells
None	0.9	34	1.2	96
0	0.6	1	1.5	2
4.5	0.4	7	1.2	5
10	2.5	39	4.4	63

* 5-Bromodeoxyuridine, 8 μg/ml; cultures have six dissociated blastoderms/ml; duplicate experiments.

younger blastoderms were used at 20 hr of incubation, selecting those that were at the definitive streak stage or younger. A higher concentration of disaggregated blastoderms had to be employed, six per milliliter, in order to get a similar concentration of cells and to get good growth. As shown in Table II, in these younger cells, BUdR at 8 μg/ml completely abolished morphological maturation and hemoglobin formation when added at the time of explantation or 4.5 hr later. However, sensitivity to the drug had greatly decreased by 10 hr after explantation, the equivalent of one cell cycle. There was no obvious effect of BUdR on cell numbers, although there was, as usual, considerable variation in the yield of erythroid cells. BUdR-treated cells look much like the usual proerythroblasts and basophilic erythroblasts, but there are subtle differences in staining characteristics which we have not studied further. In these experiments no special precautions were taken to avoid exposing the cells to visible light, although most of the time they were in a dark incubator. Visible light is known to induce mutations in cells which have the BUdR in their DNA.

DISCUSSION

These suspension cultures make it much easier to study erythroid cell differentiation. They do as well as the intact embryo for the first day as far as cell cycle times are concerned. They undergo up to seven doublings in cell numbers between day 1 and day 4, which is much better than had been obtained for organ cultures of comparable age (Reynolds and Ingram [23]) but is still much less than the numbers found in the intact embryo (18×10^6 cells on day 4; G. A. Bruns [4]). Presumably there is very little recruitment into the erythroid pool in our cultures, although more than in the organ cultures of Hagopian and Ingram (8).

It appears that the entrance of precursor cells into a particular recognizable and determined pathway of differentiation can be prevented by BUdR without affecting the rate of cell division and also reversibly (1, 3, 6, 7, 11, 13, 19, 26, 32). The fact that reversal can be obtained with an ex-

cess of thymidine is interesting, but does not at this stage define the mechanism of action of BUdR. The partial reversal obtained by addition of thymidine after 21 hr of culture (Table I) can also be interpreted as complete reversal with differentiation starting 21 hr later than in the control. For example, in Table I, one might compare the 56% benzidine-positive cells obtained on day 3 after such late thymidine reversal with the 47% and 64% found a day earlier in the two control cultures. Such treatment, in vitro, might make possible a biochemical study of the molecular events of entry into recognizable differentiation and hence has been the object of intensive study in several laboratories. In particular, in vitro systems of myogenesis, pancreatic enzyme formation, liver enzyme formation, erythropoiesis, and neuronal cell differentiation have been studied. To date, the data have been puzzling and no good hypothesis of BUdR action has been put forward. Although it is known that BUdR will be incorporated into DNA in place of thymidine and may then act as a mutagen, there is no reason to suppose that this is how it acts in stopping differentiation The ready reversibility by thymidine speaks against such a mechanism of action In fact, it has been suggested (26) that DNA synthesis is not necessary for the effect on the differentiation of neuroblastoma cells in vitro, but this happens to be an exceptional observation in that BUdR stimulates differentiation of these cells. On the other hand, it has also been suggested (31) that the mechanism of BUdR suppression of differentiation (and its reversal) necessarily involves DNA synthesis More experiments are certainly needed to settle this issue and also to decide whether BUdR acts before or only after a cell line has become determined.

Erythropoietic differentiation, however, follows the more common pattern and is suppressed by BUdR. It is interesting that our suspension cultures were much more sensitive to BUdR than the organ cultures of Miura and Wilt (19). However, the lower concentration used is in line with those employed by others for cell cultures.

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