CULTURE OF ERYTHROPOIETIC CELLS FROM CHICK BLASTODERMS

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Erythropoietic cells from early chick embryos have been successfully maintained in vitro both in tissue culture (Murray, 1932; Settle, 1954; O'Brien, 1960; Spratt and Haas, 1960 *a* and *b*; Hell, 1964; Levere and Granick, 1967; Wilt, 1967; Hagopian and Ingram, 1971; and Wenk, 1971) as well as in suspension culture (Hagopian et al., 1972). In all cases potential erythropoietic cells will continue to differentiate into mature erythrocytes during the culture period. The present communication described a technique which provides clean cultures of almost pure populations of erythroid cells from chick blastoderms. Some preliminary experiments using the drugs actinomycin D and cordycepin are also reported.

MATERIALS AND METHODS

Fertilized eggs of the White Leghorn breed were obtained from the Spafas Co., Norwich, Conn.: sera and antibiotics were purchased from the Grand Island Biological Co., Grand Island, N. Y. Actinomycin D is obtained from Calbiochem, La Jolla, Calif., and cordycepin from Sigma Chemical Co., St. Louis, Mo. 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 U/ml, 100 μg/ml, and 0.25 mg/ml, respectively).

After incubation, usually for 24 h, the blastoderms were removed in Howard's Ringer solution, cleaned of as much yolk as possible, and transferred to medium. All blastoderms were carefully examined and their developmental stages determined before use. Unless otherwise indicated, only definitive streak and head process stages (stages 4 and 5 according to New, 1966) were used. The entire potential erythropoietic area, as mapped by Settle (1954), were carefully excised using steel needles and collected in some culture medium. The desired number of tissue areas, one from each blastoderm, were transferred to a 35 × 10 mm Falcon plastic Petri dish (Falcon Plastics, Division of B-D Laboratories, Inc., Los Angeles, Calif.) which contains fresh medium and the tissues were dissociated mechanically by pumping up and down through a Pasteur pipette which is drawn out to a tip diameter of 0.5 ± 0.1 mm. For all experiments, except as noted below, six erythroid areas were dissociated in 0.5 ml of medium. 0.1 ml aliquots of the fine suspension were placed in each chamber of an eight-chamber Labtek tissue culture chamber/slide unit (Division Miles Laboratories, Inc., Westmont, Ill.). The culture was incubated inside a glass Petri dish containing cotton wool moistened with water or Ringer's solution at 39.5°C without shaking in a water saturated air atmosphere. When larger cell numbers were desired for obtaining sufficient hemoglobin for electrophoresis, 1 ml of the cell suspension was cultured in a 35 × 10 mm Falcon plastic Petri dish.

The changes in cell number and morphology were monitored by preparing slides from the cultures according to the technique described by Hagopian et al. (1972). The slides were stained with benzidine for hemoglobin and counterstained with Wright's Giemsa. Since these are stationary cultures, the culture vessel was swirled gently several times to resuspend unattached cells before a sample is taken.

For the analysis of hemoglobin produced by cells in culture, 1 ml cultures in 35 × 10 mm Falcon plastic Petri dishes were used. The culture medium containing unattached erythroid cells was collected, the bottom of the dish was rinsed two times with ice-cold Howard's Ringer, and the cell suspensions were pooled. The cells were then washed three times with ice-cold Howard's Ringer by gentle centrifugation.

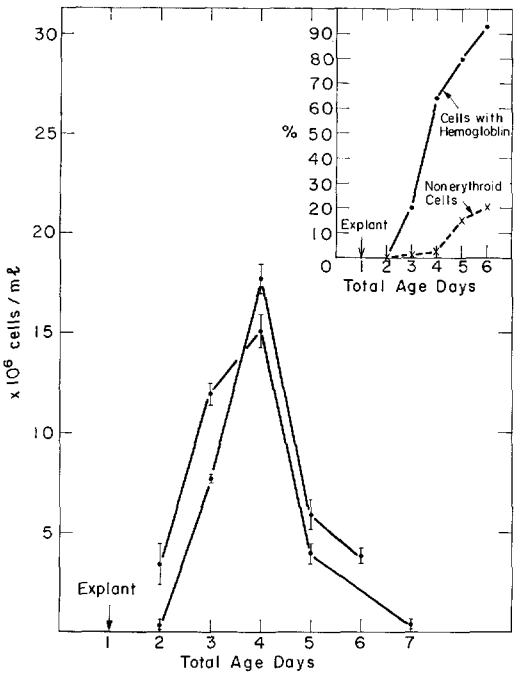

FIGURE 1 Yield of total suspended erythroid cells in culture. The solid lines connect the means of two separate cultures. *Insert* shows the proportion of hemoglobin containing cells as a percent of total erythroid cells and of nonerythroid cells as a percent of total cells in culture.

The packed cells were lysed in 30–50 μl of 0.001 M phosphate buffer (pH 7.6), left to stand at room temperature for 15 min, then mixed vigorously with 10 μl toluene and 15 μl carbon tetrachloride, and centrifuged. Ferricyanide was added to the clear lysate to convert the hemoglobin to the methemoglobin form (Moss and Ingram, 1968); the lysate was then applied to polyacrylamide gels for electrophoresis (Moss and Ingram, 1968).

RESULTS AND DISCUSSION

A typical growth curve of erythropoietic cells from definitive streak/head process embryos is shown in Fig. 1. The cells increased in number rapidly during the first 2 days of culture, reached a maximum on the 3rd day, and decreased sharply thereafter. By the 6th day of culture, very few erythroid cells remained.

Hemoglobin was first detectable in the cells by benzidine staining on the 2nd day of culture. By the 4th day, between 80% and 90% of the erythroid cells contained hemoglobin (Fig. 1).

The main advantage of this culture technique is that is provides clean, homogeneous, and concentrated cultures of chick erythroid cells. This is accomplished first by using only cells from the potentially erythroid regions of the blastoderm as the starting material, and second, by using stationary culture in order to encourage the attachment of nonerythroid cells (mainly fibroblastic

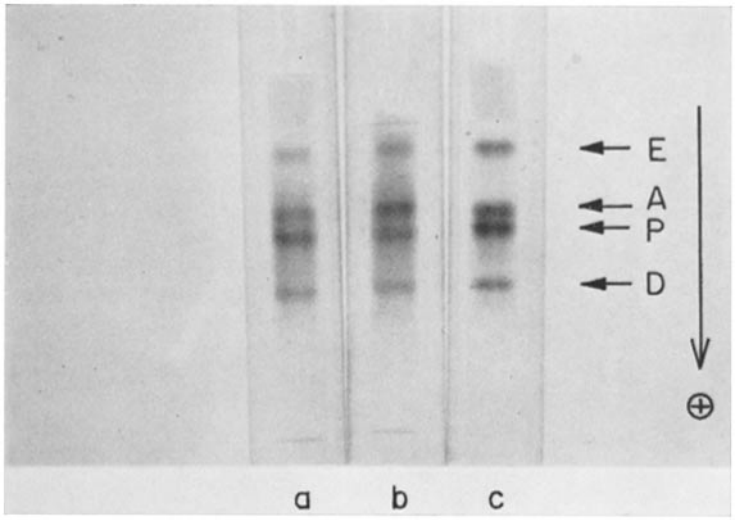

FIGURE 2 Polyacrylamide gel electrophoretic patterns of hemoglobins extracted from cultured erythroid cells from definitive streak blastoderms. (*a*) Hemoglobins from cells after 3 days of culture in 50 μg/ml cordycepin. (*b*) Hemoglobins from cells after 3 days of culture in control medium. (*c*) Standard hemoglobin solution from incubating erythroid cells from 7-day embryos.

cells) to the bottom of the culture vessel. Since erythroid cells become progressively less adhesive during the course of differentiation, cells harvested from the medium contain almost pure populations of erythroid cells during the first 3 days of culture (Fig. 1) showing the expected morphological characteristics. The advantages of these cultures are especially significant in view of the fact that biochemical experiments are now made feasible. The reduction of contaminating yolk material and nonerythroid cells as well as the high cell-to-medium ratio in these cultures allow the efficient use of radioactive isotopes. Thus, the macromolecular events which take place in the cells during differentiation can be studied.

Polyacrylamide gel analysis of the hemoglobin produced by the cells on the 3rd day of culture showed that all four types of hemoglobin were present (Fig. 2 *b*). This result is similar to those observed by Hagopian and Ingram (1971) in tissue culture, and Hagopian et al. (1972) in shaking suspension cultures of chick blastoderms. The morphology of cells in stained preparations (Bruns, 1971) makes it quite clear that erythroid cells of both the primitive and the definitive series are present by the 4th day of culture. As compared to the normal sequence of events that occur *in ovo* (Bruns, 1971), the production of the hemoglobins E and P by cultured cells, which are characteristic of primitive cells, is delayed by approximately 40 h, while the hemoglobins D and A, characteristic of the definitive cells, appear at least 2–3 days earlier than *in ovo*. These temporal alterations in cultured cells are not surprising, since the cells are placed under conditions much different from those *in ovo*.

Actinomycin D, at concentrations ranging from 0.05 μg/ml to 2.0 μg/ml, was tested for its effect upon cultures derived from erythroid areas from primitive streak, definitive streak, head process, and head fold stages of development. It was found that even the lowest concentration tested (0.05 μg/ml) when added to initial cultures caused all erythroid cells to die within 24 h of culture, regardless of the developmental stage of the embryos from which the cells were obtained. However, if the cells were allowed to grow for some time before actinomycin D was added (at 0.2 μg/ml), some erythroid cells survived. The results of such an experiment using cells from definitive streak embryos are shown in Table I. When actinomycin D was added after only 8 h of culture, no erythroid cells remained by 48 h of culture. If actinomycin D was added at 16 h of culture, some erythroid cells did survive until 72 h of culture, but none of them contained detectable amounts of hemoglobin. However, if actinomycin D is added after 24 h of culture or later, those erythroid cells which survive do contain detectable amounts of hemoglobin by 72 h of culture. Later addition of actinomycin D had less drastic effect, but even then cell numbers decreased and the survivors had markedly fewer hemoglobin-containing cells.

We conclude that resistance to actinomycin D with respect to survival and multiplication of cells and with respect to hemoglobin synthesis is gradu-

TABLE I

Effect of 0.2 μg/ml Actinomycin D Added to Cells from Definitive Streak Embryos after Various Times of Culture

Time of culture (h)	48 h culture		72 h culture	
	No. of erythroid* cells/ml	% of cells with hemoglobin	No. of erythroid* cells/ml	% of cells with hemoglobin
Act. D added				
8	0	0	0	0
16	0.4×10^6	0	5.4×10^2	0
24	6.9×10^6	0	2.8×10^6	19
34	6.0×10^6	0	7.7×10^6	15
48	20.5×10^6	1	11.9×10^6	18
No act. D added (control)	18.0×10^6	7	32.0×10^6	53

* Erythroid cells not containing hemoglobin were identified as basophilic erythroblasts by morphologic criteria.

TABLE II

Effect of Cordycepin When Added to Initial Cultures of Cells from Definitive Streak and Intermediate Streak Embryos

Stage of embryos	Conc. cordycepin	48 h culture: No. of erythroid cells/ml	48 h culture: % of cells with hemoglobin	72 h culture: No. of erythroid cells/ml	72 h culture: % of cells with hemoglobin
Definitive streak	0 (control)	12×10^6	9	32×10^6	53
	50 μg/ml	9.9×10^6	15	14.2×10^6	60
	100 μg/ml	1.2×10^6	30	2.3×10^6	100
Intermediate streak	0 (control)	0.9×10^6	17	0.8×10^6	73
	100 μg/ml	0.2×10^6	67	0.05×10^5	86

ally acquired by the cells as they mature, particularly on and after 24 h of culture. Presumably, at any one time, the population contains both more and less mature cells with more or less resistance to actinomycin D. The data also indicate that at least partial sensitivity of hemoglobin synthesis to the inhibitor remains after sensitivity of cell multiplication to the inhibitor has ceased, as indicated by the lower proportion of hemoglobinized cells among the survivors when actinomycin is given at 24, 34, or 48 h of culture. On the other hand, it is likely that at least some of the messenger RNA for globin is present before 24 h of culture. The earliest time when hemoglobin is detectable in the cultured cells by staining is at 48 h of culture. This suggests that either the messenger RNA for globin is stable during the 24 h period between its synthesis and its translation, or that globin is made during the 24 h but the formation of hemoglobin occurs later. These results are consistent with those observed in organ cultures by Wilt (1965).

Chick erythroid cells in culture are relatively insensitive to the drug cordycepin (3′-deoxyadenosine) (Table II) while human fibroblasts in culture are killed rapidly by 20 μg/ml of cordycepin. When cordycepin is added at a concentration of 100 μg/ml to initial cultures, cell numbers are significantly reduced after 24 h of treatment, but those cells which survive will produce detectable amounts of hemoglobin by 48 h of culture: in fact a higher proportion of the survivors are hemoglobinized in contrast to the actinomycin results. This is observed even with cells obtained from embryos as young as the intermediate streak stage (stage 3, according to New, 1966). The hemoglobins produced by cells from definitive streak embryos after 72 h of culture in 100 μg/ml cordycepin were analyzed by polyacrylamide gel electrophoresis (Fig. 2 *a*) and all four types of hemoglobins are seen to be present in the treated cultures as well as in the controls. This indicates that cordycepin does not act selectively upon one of the two different erythroid cell types. However, one can propose the hypothesis that cordecypin does selectively kill the less advanced cells in the original explant. If this is so, then it is further indicated that the primitive and definitive cell series develop simultaneously in culture and not consecutively as *in ovo* (Bruns, 1971). The reason for the relative insensitivity of chick embryonic erythroid cells in culture to cordycepin is not known. The possibility that cordycepin is not absorbed into the erythroid cells is unlikely, since cell numbers are significantly reduced and cell morphology is af.ected.

SUMMARY

A culture technique is described which yields clean and almost pure populations of embryonic chick erythroid cells. These cells continue to differentiate into mature erythrocytes in cultures and thus provide a useful system for the biochemical analyses of macromolecular events that occur during erythroid differentiation.

Erythroid cells from definitive streak embryos are able to synthesize hemoglobin in the presence of 0.2 μg/ml of actinomycin D when the drug was added after 24 h of previous culture, suggesting that at least some globin messenger RNA is synthesized before 24 h of cultue. Hemoglobin is first detected in cultured cells at 48 h of culture, sug-

gesting a delay before translation begins. Chick embryonic erythroid cells are relatively insensitive to cordycepin. At 100 μg/ml, cell numbers are significantly reduced, but a higher proportion of surviving cells produce hemoglobin.

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