STIMULATION OF HEME ACCUMULATION AND ERYTHROID COLONY FORMATION IN CULTURES OF CHICK BONE MARROW CELLS BY CHICKEN PLASMA

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ABSTRACT

A fibrin clot culture system with high plating efficiency is described for the growth of erythroid cells from chick bone marrow. Erythroid colonies grown in the absence of adult chicken plasma (spontaneous colonies) were either benzidine-negative or weakly benzidine-positive. Colonies grown in the presence of chicken plasma were 90% strongly benzidine-positive and 40% more abundant than spontaneous colonies. Plasma from anemic chickens was more effective than control plasma in inducing heme accumulation (heme-stimulating activity) and in increasing the number of erythroid colonies (colony-stimulating activity). Spontaneous colonies from 48-h cultures were transformed into benzidine-positive colonies by exposing them for 6–10 h to chicken plasma.

KEY WORDS erythroid · colony formation · differentiation · anemic plasma · fibrin clot · chick marrow

Successful cloning of erythroid cells in plasma clot culture has been reported for mouse (2, 3, 4, 9), rabbit (8), sheep and goats (1), humans (10), and chickens (7). In all cases, anemic plasma preparations with in vivo erythropoietin activity induced both erythroid proliferation and maturation when added to the culture medium in vitro.

The existence of a humoral factor which controls the in vivo differentiation of avian erythroblasts was demonstrated by Rosse and Waldman (5) and confirmed by Rusov (6). With the use of in vivo assays, 1.5-fold (5) and 1.4-fold (6), increases in avian erythropoietic activity in anemic chicken plasma as compared with control chicken plasma were reported. Samarut and Nigon (7) showed the induction of chicken erythrocytic colonies by some factor present in anemic chicken serum, but not in control chicken serum. By analogy with the mammalian systems, in which fractions from anemic plasma with in vivo erythropoietin activity have been used to promote erythropoietic differentiation in vitro, the authors suggested that this in vitro effect could be due to an avian erythropoietin.

Mammalian erythropoietin was found to have no effect on cultures of chicken bone marrow (7), confirming the difference between hypothetical avian erythropoietin and mammalian erythropoietin as demonstrated in vivo by the earlier work of Rosse and Waldman (5). These findings suggested the necessity to characterize avian erythropoietin as part of an overall effort to elucidate the factors that control avian erythroid proliferation and differentiation in vitro.

In the studies to be discussed in this paper, we developed a new method for cloning chick erythroid cells with high plating efficiency, and we studied the effects of anemic and control chicken plasma on erythroid cell proliferation and maturation.

MATERIALS AND METHODS

Roosters were purchased from SPAFAS (Norwich, Conn.) and made anemic by injections of acetophenylhydrazine (Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N. Y.). The birds were injected intramuscularly with 2–3 ml of 1.5% acetophenylhydrazine/kg of body weight daily for 5–8 days. Birds were bled by the jugular vein and the blood was collected in Ringer's heparinized solution. Plasma was obtained by centrifugation at 1,000 g for 10 min and kept frozen at −20°C.

The plasma clot technique of Stephenson et al. (9) was modified for the culture of chick bone marrow erythroid cells. Bone marrow was obtained from the femurs of 2-day-old chicks (SPAFAS). Both ends of the femur were cut off and the cells were removed under sterile conditions by flushing the marrow cavity with NCTC-109 medium (Microbiological Associates, Bethesda, Md.) with a syringe. Clumps of cells were dissociated by passing the suspension through a 20-gauge needle. The cell concentration was determined with a hemocytometer and adjusted to 5×10^6 cells/ml. Mature erythrocytes, distinguished by their oval shape, were not included in the counts.

The cell suspension contained: 55 μl of NCTC 109; 20 μl of fetal calf serum; 10 μl of 6% bovine serum albumin; 3 μl of 40 mg/ml of fibrinogen; 1.5 μl of 1 M HEPES, pH 8.0; 1.5 μl of 5% sodium bicarbonate; 1 μl of 0.042 M β-mercaptoethanol; 0.5 μl of 5 mg/ml gentamicin, and 10 μl of the above chick bone marrow cells in NCTC-109. The volume of chicken plasma or sterile water was no greater than 5% of the total final medium. Fetal calf serum was obtained from Grand Island Biological Co. (Grand Island, N. Y.). Bovine serum albumin was obtained from Sigma Chemical Co. (St. Louis, Mo.) and was detoxified with AG-501 8D resin, adjusted to 6% concentration in phosphate-buffered saline at pH 8.0, filtered, and kept frozen. Lyophilized human fibrinogen was obtained from A. B. Kabi (Stockholm, Sweden) and reconstituted in NCTC-109 just before use. Gentamicin was from Schering Corp. (Kenilworth, N. J.). Topical thrombin was from Parke, Davis & Co. (Detroit, Mich.) (25 NIH U/mg).

A piece of plastic containing 24 wells (model MRC-96, Linbro, Hamden, Conn.) was put into each 100 × 20 mm Petri dish containing 2–3 ml of sterile water. Thrombin was added to each well at 0.2–0.4 NIH U/well in 1 μl of volume, and the final culture medium was made by pipetting 100 μl of cell suspension into each well. The dishes were incubated in a water-jacketed incubator at 39°C in water-saturated atmosphere containing 2.5% CO_2 in air. To measure the pH of the medium during incubation, 1-ml aliquots of cell suspension were incubated in plastic tubes. From these measurements the pH of the medium was kept at 8.0 by small adjustments of the percentage of CO_2 in the incubator.

The plasma clots were removed from the wells onto a slide and immediately dehydrated, fixed, and stained by the benzidine-hematoxylin method, as described (4).

Colonies containing more than eight cells were scored at × 100. Erythroid colonies were easily recognized by small cell size, general morphology (Fig. 2), and the central spherical nuclei.

All cells in each colony had similar morphologic and staining characteristics. Erythroid colonies, identified by their morphology, were classified in two groups either as benzidine-negative and weakly benzidine-positive or as benzidine-positive, as judged by the staining properties of the cytoplasm. Benzidine-negative cells stained blue, whereas benzidine-positive cells stained intense orange. In intermediate cells weakly stained by benzidine, the cytoplasm was light brown. Only colonies containing strongly benzidine-stained cells were scored as benzidine-positive colonies. The few nonerythroid colonies found (granulocyte and macrophage colonies) were recognized by the large cell size and peripheral nuclei of their constituent cells.

RESULTS

Growth Characteristics of Chick Erythroid Colonies

To study the effect of pH in chick erythroid cell growth, separate chick bone marrow cultures were set up, each supplemented with different amounts of sodium bicarbonate. The pH of the culture medium allowing erythroid cell growth in the presence or absence of chicken plasma was narrow: 8 ± 0.2 (Fig. 1). No erythroid colonies were obtained at about pH 7.4, either in the bovine plasma/beef embryo clot (Fig. 1) or in the fibrinogen/thrombin clot (data not shown). The relatively small number of nonerythroid colonies seen at pH 7.4 decreases >50% at pH 8.0.

Maximal plating efficiency of erythroid colonies was obtained at the concentrations of the components of the culture medium listed in Materials and Methods. The maximum plating efficiency as a function of the concentration for each component was within a narrow range of values (data not shown). Fetal calf serum and bovine serum albumin batches were pretested to obtain optimal growth of the colonies.

Bovine plasma and beef embryo extract (7, 9) were replaced by fibrinogen (1–2 mg/ml) and thrombin (2–4 NIH U/ml). The concentration of these two components was not critical within these ranges, but thrombin had to be kept below 8 NIH U/ml (0.8 NIH U/well) to avoid toxic effects. The fibrin clot forms in ~30 s, which was quicker than the bovine plasma system. The fibrin clot culture also allowed the assay of substances at concentrations that prevented bovine plasma from

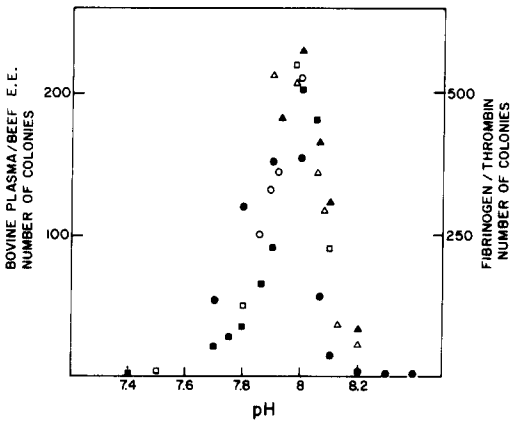

FIGURE 1 Dependence of spontaneous chick erythroid colonies on pH. The experiments were performed by including increasing amounts of sodium bicarbonate in the final medium. The pH was measured at the end of 48 h of incubation with a microelectrode immediately after the cultures were removed from the incubator. Other details were as described by MacLeod et al. (4). ●—●, bovine plasma/beef embryo extract in 5% CO_2, BSA, and HEPES at pH 8.0, incubation at 37°C. ○—○, bovine plasma/beef embryo extract in 2.5% CO_2, BSA, and HEPES at pH 8.0, incubation at 37°C. ■—■, bovine plasma/beef embryo extract in 5% CO_2, BSA, and HEPES at pH 7.2, incubation at 37°C. □—□, bovine plasma/beef embryo extract in 2.5% CO_2, BSA, and HEPES at pH 8.0, incubation at 39°C. ▲—▲, bovine plasma/beef embryo extract in CO_2, BSA, and HEPES at pH 8.0, incubation at 39°C. △—△, fibrinogen/thrombin in 2.5% CO_2, BSA, and HEPES at pH 8.0, incubation at 39°C.

clotting and had a greater and more reproducible plating efficiency (7).

Under the conditions described, spontaneous erythroid colonies after 2 days of incubation were either benzidine-negative (Fig. 2*A*) and stained blue or weakly benzidine-positive and stained light brown. Spontaneous erythroid colonies were obtained in cultures of bone marrow from chick as well as from adult chickens, and they did not require chicken plasma factors to grow. The number of spontaneous colonies increased during the first 45–60 h of culture and decreased thereafter (Fig. 3 A). The same number of spontaneous colonies was observed after 2 days of incubation by adding NCTC 109, sterile water, or nothing to the culture medium.

When anemic chicken plasma was included at the onset of the cultures, 90% of the colonies stained intense orange (benzidine-positive colonies) after two days of incubation (Fig. 2 B). The intensity of the benzidine stain was maximal at 4 days in culture. The maximum number of benzidine-positive colonies was found at 45–50 h in culture. This number was 40–50% greater than the maximum number of spontaneous colonies (Fig. 3 B). Therefore the addition of anemic chicken plasma caused both a change in the staining properties and an increase in the number of erythroid colonies.

Fig. 2 shows the morphology of benzidine-negative (*A*) and benzidine-positive (*B*) erythroid colonies obtained in the presence of anemic chicken plasma. Big nuclei, cytoplasmic shape, and blue-stained cytoplasm were characteristics of

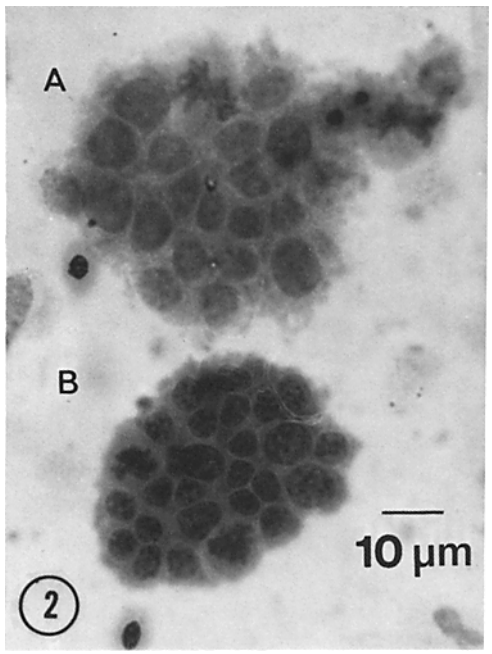

FIGURE 2 Morphology of benzidine-negative (*A*) and benzidine-positive (*B*) chick erythroid colonies. Chick bone marrow cells were incubated in the presence of anemic chicken plasma for 48 h in fibrin clot cultures. Under these conditions, only 5–10% of the erythroid colonies were benzidine-negative or weakly benzidine-positive and the rest were strongly benzidine-positive. Fixing and staining were carried out as described by the benzidine-peroxide-hematoxylin method. The cells on the upper colony (*A*) are immature erythroid cells as denoted by the large nuclei and the blue-staining cytoplasm (light in the figure). The cells on the lower colony (*B*) are more mature showing condensed nuclei and orange-stained cytoplasm (dark in the figure). All the cells within a colony show nearly the same degree of maturation. × 800.

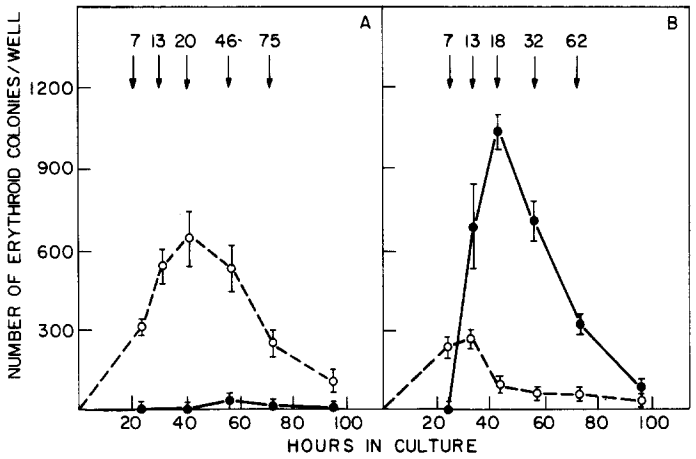

FIGURE 3 Time-course of the number of erythroid colonies produced in the absence (A) and in the presence (B) of anemic chicken plasma. Cultures were set up as described in Materials and Methods at 50,000 chick bone marrow cells/well. Cultures were harvested, fixed at the times indicated in the figure, and stained simultaneously. Only colonies containing strongly benzidine-positive cells were scored as benzidine-positive colonies. Two wells were scored separately; the averages and ranges are represented in the figure. Numbers above the arrows indicate the average colony size calculated from the sizing of 60 colonies per time-point at × 400. (A) Cultures with 4 μl of sterile water/well. (B) Cultures with 4 μl (128 μg of protein) of anemic chicken plasma/well. ○, benzidine-negative and weakly benzidine-positive erythroid colonies. ●, benzidine-positive colonies.

the more immature erythroid colonies (benzidine-negative colonies). Condensed nuclei, absence of cytoplasmic projections, and intense orange-stained cytoplasm were characteristics of the more mature erythroid colonies (benzidine-positive colonies). Colonies with intermediate characteristics of those shown in the figure were abundant in cultures made in the absence of anemic chicken plasma. Fig. 2 also illustrates the typical homogeneity in the degree of maturation of the erythroid cells from the same colony. 1–5% of the total number of colonies were nonerythrocytic under both experimental conditions (see Materials and Methods).

No significant differences in the average size of the erythroid colonies were observed between spontaneous and benzidine-positive colonies within each experiment. Both types of erythroid colonies showed doubling times of 9–11 h, as calculated from the average colony sizes throughout the culture period (Fig. 3).

To further characterize the fibrin clot culture system, the relation between the number of erythroid colonies produced at 48 h in culture and the number of cells plated in the presence and absence of anemic chicken plasma was determined. A strong correlation exists between the number of benzidine-positive colonies and the number of plated cells up to 50,000 cells/well. In the absence of plasma, however, cells plated at <10,000 cells/well form no or very few colonies. The ratio between benzidine-positive colonies and spontaneous colonies was nearly constant over concentrations of 20–50,000 cells/well. Heme accumulation and increase in the number of erythroid colonies were stimulated by the addition of anemic chicken plasma to the cultures, no matter how many cells were plated (Fig. 4). In bone marrow from anemic chicks the same observations were made, but the number of benzidine-positive colonies increased considerably, even though there was no change in the number of spontaneous colonies (Fig. 4).

The effect of the dosage of control and anemic chicken plasma on the heme content and the number of erythroid colonies produced at 48 h in culture was studied. The intensity of the benzidine stain in the erythroid colonies was dependent on the concentration of chicken plasma added. At low dosages, erythroid colonies were benzidine-negative or slightly benzidine-positive. At higher dosages, 90–95% of the erythroid colonies were benzidine-positive. Moreover, the intensity of the benzidine stain increased as the chicken plasma concentration was increased in the culture. On the other hand, the number of benzidine-positive

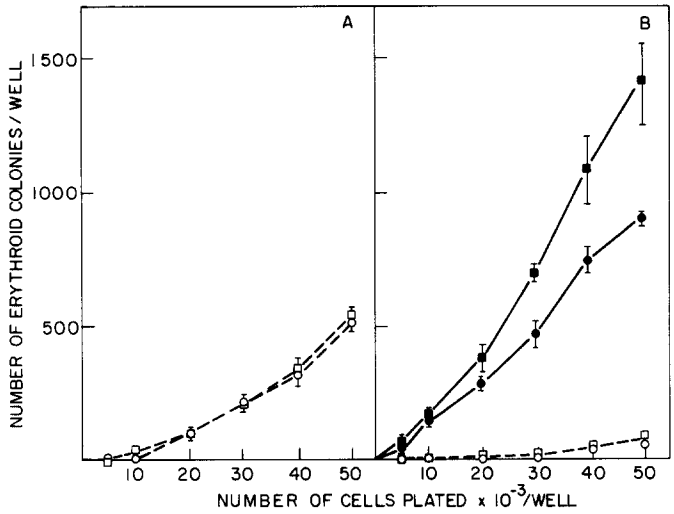

FIGURE 4 Relation between the number of control or anemic bone marrow cells plated and the number of erythroid colonies produced at 48 h in culture in the absence (A) and presence (B) of anemic chick plasma. Chicks were bled from the wing vein, about 15% of their total blood volume. Separate cultures were set up the following day, using bone marrow from the anemic chicks and from control chicks. Two wells were scored separately; the averages and the ranges are represented in the figure. (A) Number of spontaneous colonies produced by incubation of control (○) and anemic (□) chicken bone marrow with 4 μl of sterile water/well. (B) Number of benzidine-positive (●, ■) and benzidine-negative (○, □) colonies produced by incubation of control (●, ○) and anemic (■, □) chicken bone marrows with 4 μl (128 μg of protein) of anemic chicken plasma/well.

colonies was equal to or less than the number of spontaneous colonies at low dosages. Further increase in the number of benzidine-positive colonies up to 50% of the spontaneous colonies was dependent on higher concentrations of chicken plasma (Fig. 5). Plasma from chickens made anemic by acetophylhydrazine injections was more effective than control chicken plasma, inducing 1.7-fold more benzidine-positive colonies (Fig. 5). The addition of mammalian erythropoietin (Connaught Medical Research Laboratory, Ontario, Canada) did not have any effect on the chick bone marrow cell culture up to 1.4 U/ml.

Evidence for Separable Heme and Colony Stimulations

The average size, the change in staining properties, and the increase in the number of the erythroid colonies were studied by adding anemic chicken plasma to the cultures at different times during incubation and harvesting the cultures after 50 h of incubation (Fig. 6). The average colony size did not vary in response to the addition of anemic chicken plasma at different times during the incubation period. During the first 8–10 h of culture, the capacity of the chick bone marrow cells to respond to the addition of anemic chicken plasma and to form benzidine-positive colonies decreased very quickly. Thereafter, the addition of anemic plasma did not increase the total number of colonies, but it did cause all the colonies to be benzidine-positive at 50 h. The addition of anemic plasma only 7–9 h before harvesting did not produce either an increase in the number of colonies or a change to benzidine-positive colonies.

90% of the spontaneous colonies obtained by 48 h of incubation in the absence of anemic chicken plasma appeared as benzidine-positive colonies after the addition of anemic chicken plasma and further incubation overnight. Parallel cultures incubated in the absence of anemic chicken plasma contained only benzidine-negative colonies and slightly benzidine-positive colonies.

To study the time-course of the conversion of the spontaneous colonies into benzidine-positive colonies, anemic chicken plasma was added to the cultures after 48 h of incubation, and the cultures were reincubated and harvested at different times after the addition. No benzidine-positive colonies were induced during the first 4 h after the addition of anemic chicken plasma. The time at which the spontaneous erythroid colonies be-

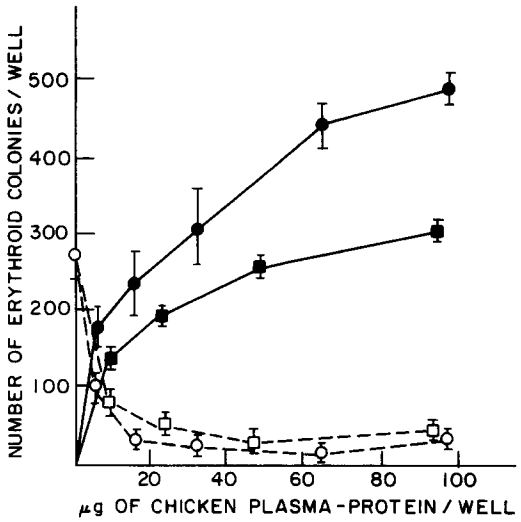

FIGURE 5 Effect of the dosage of control and anemic chicken plasma on the number of erythroid colonies produced at 48 h in culture. Conditions of culture were as described in Materials and Methods, using 50,000 cells/well from chick bone marrow. Chick plasma (0.2–3 μl/well) was added at the onset of the cultures. Two wells were scored separately; the averages and the ranges are represented in the figure. ●, benzidine-positive colonies and ○, benzidine-negative and weakly benzidine-positive erythroid colonies induced by anemic chicken plasma. ■, benzidine-positive colonies and □, benzidine-negative and slightly benzidine-positive erythroid colonies induced by control chicken plasma.

came benzidine-positive was between 6 and 10 h, depending on the experiment (Figs. 6 and 7). The total number of erythroid colonies decreased or stayed constant during the conversion, as expected from the time-course curves (Fig. 3 A). The average size of the erythroid colonies increased during the conversion from 20 to 33 cells per colony, as expected from the division rate (Fig. 3).

DISCUSSION

The studies reported here demonstrate that chick bone marrow cells can form erythroid colonies when incubated under optimal culture conditions. Spontaneous erythroid colonies were produced in the absence of chicken plasma, in chick as well as in adult bone marrow. The factors contained in fetal calf serum or in bovine serum albumin seem to be sufficient to allow growth of these colonies. It is also possible that the cells conditioned the culture medium by producing the necessary factors, as suggested by the small number of spontaneous colonies found when cells were plated at low concentrations (Fig. 4). In contrast, Samarut and Nigon (7), using chicken bone marrow cultures, found that all the erythroid colonies were dependent on the presence of anemic chicken serum. However, they only considered as erythroid colonies those containing oval-shaped cells (mature erythrocytes). It is possible that erythroid colonies containing round-shaped cells (like some of our spontaneous erythroid colonies) would be undetected in their studies.

Both the type and the number of chick erythroid colonies in our cultures were influenced by the presence of some factor(s) contained in chicken plasma (Fig. 3). If enough chicken plasma was included in the cultures, 90–95% of the erythroid colonies appeared as benzidine-positive (heme-stimulating activity) and the total number of erythroid colonies increased 40–50% relative to the number of spontaneous colonies (colony-stimulating activity).

Heme- and colony-stimulating activities could be independently studied by adding anemic chicken plasma at different times in culture. The heme-stimulating activity was only detected after 6 h of exposure to anemic chicken plasma (Fig.

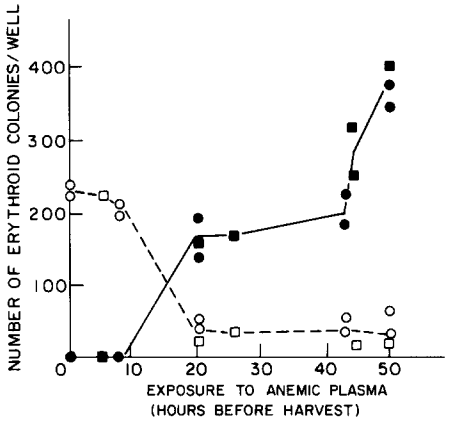

FIGURE 6 Effect of the time of addition of anemic chicken plasma on the number of erythroid colonies produced at 50 h in culture. Cultures were set at 50,000 cells/well with chick bone marrow. Anemic chicken plasma (152 μg of protein) was added at the times indicated. All the cultures were harvested after about 50 h of incubation. The average colony size was about 16 cells per colony at every point. Experiment 1: ●, benzidine-positive colonies, and ○, slightly benzidine-positive and benzidine-negative erythroid colonies. Experiment 2: ■, benzidine-positive colonies, and □, slightly benzidine-positive and benzidine-negative erythroid colonies.

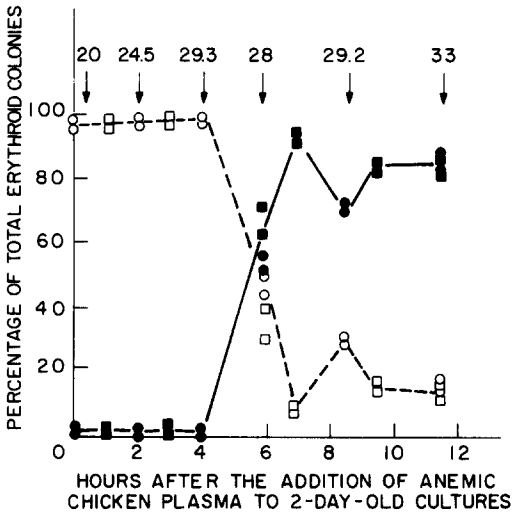

FIGURE 7 Time-course of the conversion of benzidine-negative erythroid colonies (43 h of incubation) into benzidine-positive colonies by the addition of anemic chicken plasma. Cultures were set at 50,000 cells/well as described. Incubation was allowed to proceed for 43 h, at which time (time 0 in the figure) anemic chicken plasma, 128 μg of protein, was added to every well. Cultures were reincubated and harvested at the times indicated in the figure and then stained simultaneously. The composition (expressed in percentages) of the erythroid colonies was calculated by counting 200-500 colonies at every time-point. Numbers above the arrows indicate the average colony size calculated from 30 colonies per time-point. Experiment 1: ○, slightly benzidine-positive and benzidine-negative erythroid colonies, and ●, benzidine-positive colonies. Experiment 2: □, slightly benzidine-positive and benzidine-negative erythroid colonies, and ■, benzidine-positive colonies.

7). This stimulation could be due to either the requirement for a small molecule needed for maturation, which would be transferred by a chick specific carrier (like transferrin or hemopexin), or the induction of maturation by a hormonelike molecule. The colony-stimulating activity was only detected when the anemic chicken plasma was added during the first 10 h of incubation (Fig. 6). This effect could be due to either the decay (survival factor) or the loss of inducibility (growth factor) of the colony precursors present when the marrow cells are plated.

Anemic chicken plasma showed 1.7 times more activity than control chicken plasma (Fig. 5). Both heme-stimulating and colony-stimulating activity were increased. This relative increase in activity was similar to that observed in the in vivo experiments (5, 6), suggesting that both in vivo and in vitro effects were caused by the same factor(s). However, more experimental evidence is needed to show the relation between the heme-stimulating and the colony-stimulating factor(s) on the one hand, and avian erythropoietin on the other.

Received for publication 31 May 1977, and in revised form 22 August 1977.

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