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IDENTIFICATION OF OVOTRANSFERRIN AS A HEME-, COLONY- AND BURST-STIMULATING FACTOR IN CHICK ERYTHROID CELL CULTURES

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SUMMARY

Clonal growth of erythroid cells from bone marrow of 2-day-old chicks in fibrin clots under different culture conditions has been used as independent assays for heme-, colony- and burst-stimulating activities found in anemic chicken plasma. The properties of the heme-stimulating activity analyzed by gel filtration, isoelectrofocusing, resistance to heat denaturation, ammonium sulfate precipitation, DEAE-chromatography or HAP-chromatography, suggested serum transferrin as the heme-stimulating factor(s). Heme-, colony- and burst-stimulating factor(s) from anemic chicken plasma did not separate from each other by gel filtration or isoelectrofocusing. Ovotransferrin from egg white also showed heme-, colony- and burst-stimulating activities by the assay employed even after further purification by limited trypsin digestion, electrophoresis, hydroxylapatite chromatography or fractionation by ConA-Sepharose chromatography.

Few reports exist on clonal cell culture of bone marrow erythroid cells from chicks [1, 2]. Erythroid clonal cultures have been studied in mammalian systems, mainly in the mouse or in humans [3–9].

In the mammalian systems, two kinds of erythroid precursors, DFU-E and BFU-E, have been distinguished both by their proliferative potential and their erythropoietin requirement [6]. At low concentrations of erythropoietin, small erythroid colonies are produced after 2 days in culture from CFU-E precursors. At 10-fold higher concentrations, in addition to the 2-day-old colonies, groups (bursts) of small erythroid colonies are produced from BFU-E precursors after 7–10 days in culture. In chickens, CFU-E precursors have been demonstrated [1, 2], but no BFU-E precursors have yet been described previous to the present paper.

Chick bone marrow cells will form spontaneous benzidine-negative erythroid colonies in fibrin clots when grown with fetal calf serum (FCS) [2]. The addition of chicken plasma stimulated heme accumulation (benzidine-positive colonies instead of benzidine-negative erythroid colonies) as well as a 40% increase in the total number of erythroid colonies. Because of the high level of spontaneous erythroid colonies under these conditions, the cultures can only be used to assay for heme-stimulating activity. We report here modified culture conditions which will allow both the assay of erythroid colony-stimulating activity and burst-stimulating activity.

Studies of the in vitro requirements of CFU-E or BFU-E erythroid cells show that all the mammalian cultures require the presence of both FCS and preparations with erythropoietin activity. Attempts to sub-

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stitute completely the FCS requirement have failed, although Guilbert & Iscove [10] obtained mouse erythroid cell growth (CFU-E) in a semi-defined medium containing low proportions of FCS, selenite, bovine serum albumin, human transferrin and erythropoietin. The simultaneous presence of all these components was needed in order to obtain maximal growth.

In the chick system, we have been able to completely eliminate the undefined FCS requirement, but not the bovine serum albumin requirement; a chick factor derived from chicken plasma or from egg white was also needed.

We fractionated anemic chicken plasma and assayed for three independent effects on chick erythroid cell cultures as defined previously: heme-, colony- and burst-stimulating activities. The properties for heme-stimulating activity were similar to those described for transferrin or ovotransferrin (the transferrin of egg white) and no separation of potential heme-, colony- and burst-stimulating factors could be accomplished. Commercial ovotransferrin has been found to have heme-, colony- and burst-stimulating activities.

MATERIALS AND METHODS

Heme, colony and burst assays

Bone marrow cells from the femurs of 2-day-old chicks (Spafas, Norwich, CT) were plated in the medium described in Coll & Ingram [2], except that the FCS (Grand Island Biological Co., Grand Island, NY) was omitted in some experiments, its volume being replaced by NCTC-109 (Microbiological Associates, Bethesda, MD), and that the bovine serum albumin was used at various concentrations (Sigma Chemical Co., St Louis, MO). Bovine serum albumin was reconstituted in NCTC-109 and adjusted to pH 8.0 just prior to use with freshly made NaOH. The cells were grown in fibrin clots, fixed and stained as described in detail in Coll & Ingram [2]. Erythroid colonies were recognized by the characteristic morphology of the stained cells and by their benzidine reaction or their ability to be converted to heme-containing, benzidine-positive cells by continued incubation with anemic chicken plasma.

Anemic chicken plasma was prepared by acetophenylhydrazine injections (Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, NY) as described in Coll & Ingram [2]. Heme-stimulating activity was assayed by culturing the cells in medium containing 10% FCS for 2 days, colony-stimulating activity was assayed by culturing the cells in medium containing no FCS for 2 days and burst-stimulating activity was assayed by culturing the cells in medium containing 10% FCS for 7–10 days. The rest of the experimental conditions were as indicated in the figure captions.

Protein estimations

Protein was estimated by the BioRad protein assay method (BioRad Laboratories, Richmond, CA) using bovine serum albumin as standard. The absorbance at 280 or 230 nm was used to monitor the protein concentrations of column effluents. Densitometry of Coomassie blue R-250 stained polyacrylamide gels was carried out by using a Canalco Model J densitometer.

Isoelectrofocusing

The column, model S.C. 1 of 110 ml (LKB Produkter AB, Bromma 1, Sweden) was used for the preparative isoelectrofocusing experiments as indicated by the LKB manual.

Analytical isoelectrofocusing was performed in a 0.5×25 m column. The bottom of the column was stopped with dialysis tubing and 5% polyacrylamide gel (1 cm high). After leaving the bottom of the column immersed in 2% ethylenediamine overnight, a mixture containing 1 ml of anemic chicken plasma, 25 μl of 40% BioRad ampholines (pH 3–10) and wet Sephadex G-25, was poured into the column. The top reservoir was 1% phosphoric acid as cathode solution and in the bottom reservoir was 2% ethylenediamine as anode solution. After 6 h at 200–400 V, the polyacrylamide stopper was perforated with a needle and fractions were collected by gravity elution.

Gel filtration

Sephadex (Pharmacia Fine Chemicals, Inc., Piscataway, NJ) was used for gel filtration experiments as recommended in the Pharmacia manual. Further details are given in the figure captions.

Hydroxylapatite chromatography

The gel (BioGel hydroxylapatite, BioRad Laboratories) was packed in a 3×5 cm column and equilibrated with 25 mM potassium phosphate, pH 7.4 (starting buffer).

The sample was dialysed or made up in the starting buffer and applied to the column. The eluate was collected and the column washed with starting buffer until the absorbance at 280 nm was 0. Elution of the absorbed material was done by a stepwise increase

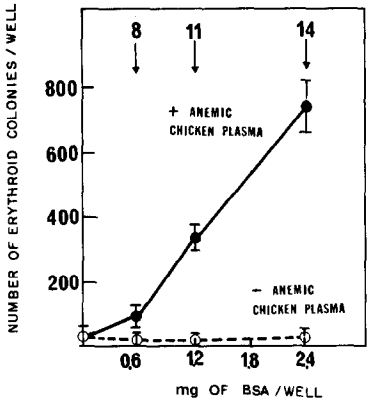

Fig. 1. Effect of bovine serum albumin (BSA) concentration upon the total number and size of chick erythroid colonies in the absence (○) and in the presence (●) of anemic chicken plasma in cultures made in the absence of FCS. Cultures were prepared as described in Materials and Methods except there was no FCS and NCTC-109 was added instead (20 μl/well). Three μl (100 μg of protein) of anemic chicken plasma/well were added to the cultures (●—●). After 2 days the cultures were fixed and stained. Each point was scored in duplicate. Averages and ranges are presented. The numbers above the arrows indicate the average number of cells/colony obtained from the sizing of 50 colonies/point.

in the buffer concentration, usually 25 mM, 50 mM, 125 mM and 500 mM potassium phosphate, pH 7.4. Three ml fractions were collected and kept at −20°C until bioassay. Assays were done using 2 μl aliquots which allowed the use of 500 mM potassium phosphate without detrimental effects to the cells in culture.

Gel electrophoresis

SDS-gel electrophoresis was performed in slabs according to the method described by Laemmli [11] except that a 5–15% gradient of polyacrylamide was used. Preparative gel electrophoresis in 160×160×3 mm slabs was performed in 7.5% polyacrylamide gels as described by Maizel [12]. Ovotransferrin from Sigma (12 mg) was added to 2 ml of distilled water, 0.2 ml of bromophenol blue, 0.4 ml of glycerol and 1.0 ml of 1 M Tris, pH 8.8, and the mixture electrophoresed. After the gel was removed from the glass plates, the two edges were cut off and stained as described by Maizel [12]. During this time the rest of the gel was frozen at −70°C. The part of the gel corresponding to the ovotransferrin was cut into small pieces and the protein was extracted in 0.1 M sodium bicarbonate: 1 M NaCl at 37°C for 4 h. The gel pieces were removed by centrifugation and the supernatant was dialysed against distilled water and lyophilized.

Trypsin-digestion of iron-saturated ovotransferrin

Two hundred mg of iron-saturated ovotransferrin (Sigma Chemical Co.) were digested for 7 h with 1 mg of trypsin (Worthington Biochemical Co.) in 4 ml of 0.1 M Tris pH 8 at 37°C with constant agitation. The digest was applied to a 2.5×33 cm column packed with Sephadex G-75, and eluted with 0.1 M sodium bicarbonate. Fractions were pooled, lyophilized and assayed.

Ammonium sulfate precipitation was performed by adding the salt in increments of 20% of saturation (at 20°C) to 10 ml of anemic chicken plasma, and adjusted to pH 6 or 8. The fractionation was carried out at pH 6 and at pH 8 with two different preparations of anemic chicken plasma. After the addition of the solid ammonium sulfate the mixture was agitated for 30 min at 20°C, followed by centrifugation. Precipitates were dissolved in distilled water and aliquots of 4 μl were assayed.

Ethanol precipitation was performed with anemic chicken plasma adjusted to pH 6. After the ethanol addition, the mixture was agitated for 30 min at 20°C, followed by centrifugation, and the supernatant fractionated with increasing amounts of ethanol. The precipitates were redissolved in distilled water and 4 μl aliquots were assayed.

Heat treatment at different pH values was carried out with anemic chicken plasma dialysed, lyophilized and redissolved in 0.1 M Hepes, pH 7.3 and adjusted to the desired pH. One hundred μl samples were heated at 60°C for 15 min. The precipitates were removed by centrifugation and 4 μl of the supernatants were assayed. No activity could be recovered from any of the precipitates.

DEAE-binding was performed with 6 ml of anemic chicken plasma diluted 1 : 1 with distilled water (same conductivity as 30 mM phosphate buffer, pH 7.4) and 3 g DEAE–cellulose (Whatman, DE-23). The mixture was adjusted to the desired pH, agitated for 30 min at room temperature and centrifuged. The pellets were eluted with 1 ml of 0.25 M NaCl, 0.05 M Na-phosphate, pH 3.5, then adjusted to pH 8, and 4 μl aliquots were assayed.

RESULTS

Small, early erythroid colonies

Cultures prepared in the absence of both FCS and anemic chicken plasma produced only a very small number of spontaneous erythroid colonies even when bovine serum albumin (BSA) was added (fig. 1). The addition of anemic chicken plasma in the amount of 100 μg of protein/well to an otherwise protein-free medium did not enable erythroid colonies to grow. However,

Table 1. *Characteristics of chick erythroid colonies grown in the presence (medium A) or in the absence (medium B) of FCS for 45 h*

Growth conditions	Properties	No additions	Anemic chicken plasma +100 μg prot/well
Medium A (+20% FCS)	Colonies/50 000 cells	740±70	925±75
	% benzidine+	4±1	95±2
	cells/colony	13.3±5	16.3±5
Medium B (−FCS)	Colonies/50 000 cells	25±5	490±30
	% benzidine+	0	85±5
	cells/colony	8±1	11.8±3

Chick bone marrow cells were grown in medium A (0.6 mg/well BSA and 0.7 mg/well FCS) or in medium B (1.8 mg/well BSA and no FCS). The other components of the medium were as described in Methods. Cultures received no protein or 100 μg of protein from anemic chicken plasma/well in 4 μl volume. Erythroid colonies containing 8 or more cells were scored by the size, central nuclei, general morphology and staining characteristics of their constituent cells, as described earlier [4]. The percentage of erythroid colonies that were strongly benzidine-positive was determined by counting about 100 colonies per point. Number of erythroid colonies and percentage of benzidine-positive were done in duplicate; averages and ranges are given. Average size and S.D. were calculated from the sizing of about 50 colonies/point.

increasing amounts of BSA to cultures containing anemic chicken plasma caused a dramatic increase in the appearance of benzidine-positive small erythroid colonies (fig. 1). Not only the number of colonies, but also the size of the colonies increased with increasing BSA concentration. In every well, 90% of the colonies were benzidine-positive.

The extent of the colony-stimulating activity by BSA varied greatly from batch to batch, even though they were all obtained from the same supplier, Sigma Chemical Corp. The optimal BSA concentration (1.5–2.5 mg of BSA/well) had to be determined for every batch. The pH of the albumin solution used is also crucial; fresh NaOH solutions must be used for neutralization, because old solutions seem to be toxic to the chick erythroid cells cultured in the absence of FCS.

In the presence of 1.8 mg of BSA/well (no FCS and no anemic chicken plasma), only a few spontaneous erythroid colonies developed, approx. 0.025 colonies per μg of BSA. The addition of FCS to these cultures stimulated the development of small, erythroid colonies to approx. 1 colony per μg of FCS protein. In cultures containing less than 0.35 mg of FCS protein/well (10%), the colonies were benzidine-negative. In cultures containing 0.7 mg or more per well of FCS, the erythroid colonies began to be slightly benzidine-positive. All these colonies could be converted into benzidine-positive colonies by the addition of 100 μg/well of anemic chicken plasma and 8–10 h of further incubation as described [2]. Anemic chicken plasma has more colony-stimulating activity than FCS and moreover produced small, erythroid colonies of which 95% were strongly benzidine-positive. By this assay, anemic chicken plasma had colony-stimulating activity to the extent of 4 colonies per μg of protein and egg white 3.8 colonies per μg of protein (data not shown). Such culture conditions can be used to estimate the stimulating activity which produces small, benzidine-negative erythroid colonies (e.g. FCS) or

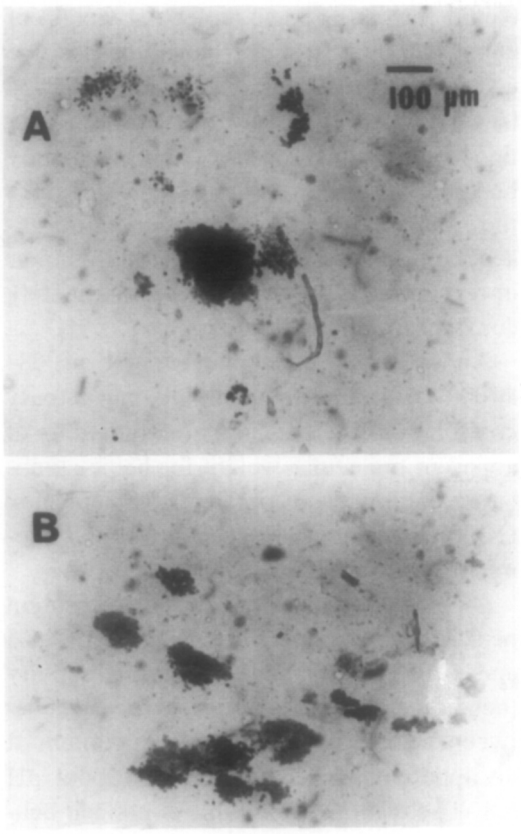

Fig. 2. Morphology and staining properties of burst-like chicken erythroid colonies. Cultures were set up as described in Materials and Methods. Chick bone marrow cells were incubated in the presence of 4 µl of anemic chicken plasma (120 µg of protein/well) for 6 days in fibrin clot cultures. Fixing and staining were carried out as described by the benzidine–peroxide–hematoxylin method. Under these conditions, burst-like erythroid colonies were either large benzidine-positive colonies (*A*) or groups of small benzidine-negative and benzidine-positive colonies (*B*). The number of burst-like erythroid colonies was 5–20/50 000 bone marrow cells. Similar colonies were absent from the cultures made without anemic chicken plasma. ×60.

the stimulating activity which produces benzidine-positive colonies (e.g. anemic or control chicken plasma).

Table 1 compares the number of erythroid colonies, the percentage of benzidine-positive colonies and the average size of the erythroid colonies obtained under different culture conditions in the same experiment. The largest number of small erythroid colonies were obtained in the simultaneous presence of FCS and anemic chicken plasma in medium containing BSA (925 colonies/well). These were also the conditions for producing colonies with the largest number of cells. In the same medium but in the absence of FCS the number of colonies was not as large (490 colonies/well) and still smaller than the number of colonies obtained by adding only FCS to the cultures (740 colonies/well). The percentage of benzidine-positive colonies was very small in cultures made in the absence of anemic chicken plasma and was about 90% in cultures made in the presence of anemic chicken plasma independently of the FCS concentration.

Cultures producing erythroid bursts

Prolonged culture of chick bone marrow cells in the presence of FCS, BSA and anemic chicken plasma showed the presence of burst-like groups of benzidine-positive erythroid colonies after 6–10 days of incubation (fig. 2). No bursts were found in cultures from which the anemic chicken plasma had been omitted. Each burst consisted of either a group of small colonies with 30–50 cells/colony or a single mass of cells containing 500 cells or more. Fig. 2 shows the morphology and staining properties of the burst-like chick erythroid colonies. Some bursts contained groups of both benzidine-positive and benzidine-negative sub-colonies. The earlier small, erythroid colonies, which were present after 2 days, had disappeared after 7 days of incubation; fibroblasts and lymphocytes were absent. Between 5 and 20 bursts were obtained from 50 000 bone marrow cells.

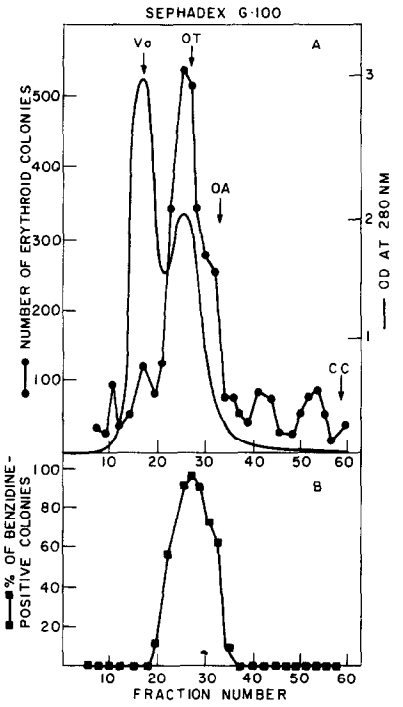

Fig. 3. Sephadex G-100 chromatography of anemic chicken plasma and assay of the fractions for chick erythroid colony-stimulating activity (*A*) and heme-stimulating activity (*B*). Anemic chicken plasma was obtained by bleeding as described. Two ml of plasma was applied to a 1.8×80 cm Sephadex G-100 column equilibrated with 0.1 M ammonium bicarbonate and eluted with this same buffer. After elution of 25 ml, fractions of 1.25 ml were collected at a rate of 7 ml/h. *Arrows: Vo,* blue dextran; *OT,* ovotransferrin; *OA,* ovalbumin; *CC,* cytochrome *c*. Six μl aliquots were assayed for colony-stimulating activity in cultures containing no FCS and 1.8 mg/well of BSA. —, OD profile at 280 nm; ●—●, no. of erythroid colonies/well; ■—■, % of benzidine-positive colonies (*B*).

Characterization of heme-stimulating factor(s) by gel filtration and isoelectrofocusing

Anemic chicken plasma was fractionated by Sephadex G-100 chromatography or by isoelectrofocusing as described in Materials and Methods. By Sephadex G-100 chromatography two protein peaks were detected in the eluate: the first peak eluted with the void volume, and the second peak eluted with an average molecular weight of about 80000. Strongly benzidine-positive colonies appeared only with fractions 23–34 (see fig. 3); the rest of the cultures contained benzidine-negative and/or slightly benzidine-positive erythroid colonies, like the control cultures incubated in the absence of added aliquots. The total number of erythroid colonies was approximately constant for all the fractions.

An apparent molecular weight of 68–80000 D for the heme-stimulating activity could be calculated. Similar estimations of apparent molecular weight for heme-stimulating activity were obtained by Sephadex G-100 chromatography of control and anemic plasma from 2-day-old chicks.

By isoelectrofocusing, two main protein peaks were obtained, focusing at pH 6.5 and pH 5.3. The heme-stimulating activity (benzidine-positive colonies) focused between pH 5.3 and 6.5 with a maximum at an apparent isoelectric point of about pH 5.8. The rest of the cultures contained benzidine-negative and slightly benzidine-positive erythroid colonies, similar to the colonies in the control cultures incubated in the absence of added aliquots. The total number of erythroid colonies was approximately constant in all the fractions.

Further characterization

Heme-stimulating activity had the following properties (table 2): it was eluted at 120 mM potassium phosphate, pH 7.4 from hydroxylapatite; it was precipitated with 80% saturated ammonium sulfate at pH 6 or with 60–80% saturated ammonium sulfate at pH 8; it was resistant to heating at 60°C and pH 6–8 but irreversibly destroyed at pH 5 or lower; it was precipitated between 25–54% ethanol at pH 6; it was bound to DEAE-cellulose at pH 9 but not at pH 7.5; and it was resistant to trypsin

Table 2. *Comparison between the properties of heme-stimulating activity and transferrins*

Properties	Heme-stimulating activity	Transferrins	Ref. (Tf or OT)[a]
Molecular weight	68–80 000 (fig. 1)	76–79 000	16 (Tf), 14 (Tf, OT)
Isoelectrofocusing	pI 5.3–6.5 (fig. 2)	pI 5.62–6.73	31 (OT)
Heat resistance	Stable at 60°C, pH 8 and 15 min Destroyed at 60°C, pH 5 and 15 min	Iron-saturated, stable at 65°C, pH 8 and 120 min Iron-free denatured at 65°C, pH 8 and 10 min	13, 32 (Tf, OT) (iron-saturated Tf and OT are converted into iron-free by exposure to pH 5)
Ammonium sulfate precipitation	pH 6, 80% saturation pH 8, 60–80% saturation	pH 6.5, 88% saturation pH 8, 65% saturation	14 (Tf) 33 (OT)
DEAE binding	Binds at pH 8, 30 mM phosphate[b] Eluted at pH 4	Binds at pH 7.4, 5 mM phosphate Eluted at pH 4.4, 75 mM phosphate	20 (OT, chicken Tf)
HAP chromatography	Eluted at 120 mM phosphate, pH 7.4	Eluted at 100 mM phosphate, pH 7.4	Author (data not shown, OT)
SDS gel electrophoresis	Active fractions possessed an 80 000 band	80 000 band	16 (Tf), 14 (Tf, OT)
Trypsin digestion	Resistant at pH 7.3	Iron-saturated are resistant Iron-free are digested	13 (Tf, OT)

[a] Tf, human serum transferrin, OT, ovotransferrin, transferrin found in the chicken egg white. Chicken serum transferrin and ovotransferrin seem to differ only in the carbohydrate composition [18–21]. Ovotransferrin is 14% of the egg white, so is the most studied transferrin, together with human serum transferrin [14].
[b] Ionic strength equivalent to 30 mM phosphate.

(80 μg of trypsin/400 μl of anemic chicken plasma, incubated at 37°C for 1 h at pH 7.3). By using purification methods in a sequential fashion (ethanol precipitation, Sephadex chromatography, DEAE–cellulose chromatography and ammonium sulfate precipitation), an active fraction with about a 30-fold purification factor was isolated. All the above-mentioned fractions possessed an 80 000 molecular weight protein band by SDS-gel electrophoresis.

Properties of heme-stimulating factor and transferrin

Table 2 shows a comparison between the properties of heme-stimulating activity and those of the ovotransferrin or serum transferrin molecules. The molecular weight estimations, the isoelectric point range, the pH dependence of resistance to heat, the ammonium sulfate concentrations needed to precipitate the factor or the transferrins, the conditions for 'DEAE binding' and the resistance to trypsin digestion are similar for the anemic chicken plasma heme-stimulating activity and for chicken ovotransferrin (iron-binding protein from chicken egg white) or human serum transferrin (iron-binding protein from human serum).

Colony- and burst-stimulating factors

Fig. 3*A* shows the protein profile and the total number of chick erythroid colonies induced by incubation of chick bone marrow cells with fractions from Sephadex G-100 chromatography of anemic chicken plasma in the absence of FCS. The number of erythroid colonies was highest (400–500 colonies/well) with fractions 25–30, whereas the rest of the cultures contained 20–70 colonies/well, like the control cultures incubated in the absence of added aliquots.

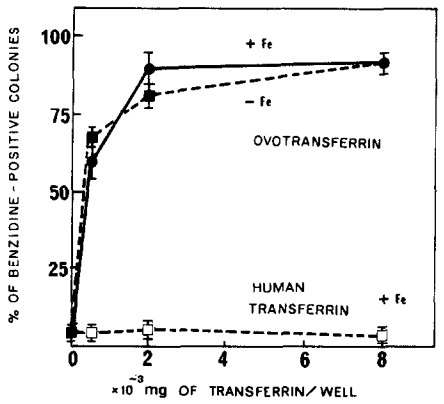

Fig. 4. Effect of the dosage of iron-saturated and iron-free ovotransferrin on the percentage of benzidine-positive colonies produced at 48 h of culture. Conditions of culture were as described in Methods, except the concentrations of FCS (0.17 mg/well) and BSA (3 mg/well). Iron-free ovotransferrin was prepared from iron-saturated ovotransferrin (Sigma) by extensive dialysis against 0.1 M Na citrate, pH 4.5 and then distilled water. Ovotransferrins were added at the onset of the cultures. Two wells were scored separately and about 100 colonies were classified as either strongly benzidine-positive or weakly benzidine-positive and benzidine-negative. Averages and ranges are given in the figure. ●—●, Iron-saturated ovotransferrin; ■---■, iron-free ovotransferrin; □---□, iron-saturated rabbit or human transferrin (Sigma).

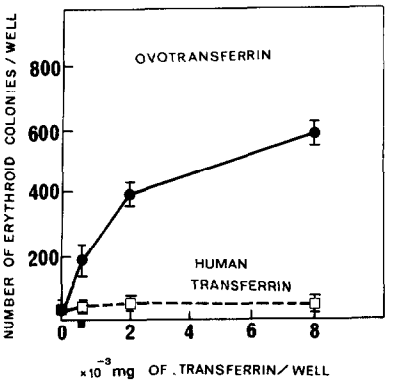

Fig. 5. Effect on the dosage of iron-saturated ovotransferrin and human transferrin on the total number of erythroid colonies produced at 48 h in cultures made in the absence of FCS. Conditions of culture were as described in Methods, except for the concentrations of FCS (0%) and BSA (1.8 mg/well). Ovotransferrin was added at the onset of the cultures. Two wells were scored separately; averages and ranges are given in the figure. ●—●, Iron-saturated ovotransferrin; □---□, iron-saturated human (or rabbit) transferrin (Sigma).

The intensity of benzidine stain was highest with the same fractions coinciding with the higher number of erythroid colonies (fig. 3*B*). The average size of the erythroid colonies was approximately constant for all the fractions. An apparent molecular weight of 68–80 000 D could be calculated for colony-stimulating activity from these data and from the elution volume of other markers.

Anemic chicken plasma was fractionated by Sephadex G-100 chromatography and the fractions were assayed for both heme-stimulating activity and burst-stimulating activity in the presence of FCS. The burst-stimulating factor was not separated from the heme-stimulating factor. Similarly, isoelectrofocusing did not separate these two activities (data not shown).

Heme-, colony- and burst-stimulating activities of ovotransferrin

Iron-saturated and iron-free ovotransferrins were obtained from Sigma Chemical Co. and assayed for heme-, colony- and burst-stimulating activities. Fig. 4 shows the effect of the dosage of iron-saturated and iron-free ovotransferrin on the percentage of benzidine-positive colonies produced at 48 h of culture. The specific activity of this preparation was 70% benzidine-positive colonies/μg of ovotransferrin.

Fig. 5 shows the effect of the dosage of iron-saturated ovotransferrin and human transferrin (Sigma) on the total number of erythroid colonies produced at 48 h in cultures in the absence of FCS. About 8–10 μg of ovotransferrin/well were required to obtain maximal numbers of erythroid colonies. This specific activity varied between 6–60 colonies/μg with different BSA batches. The erythroid colonies were ben-

Table 3. *Number of chick erythroid burst-like erythroid colonies obtained in the absence and in the presence of several concentrations of ovotransferrin*

Cultures	μg of ovotransferrin/well	No. of burst-like erythroid colonies/well
Control (no addition)	0	2.7±0.6
+ovotransferrin	0.5	8.5±0.8
	1	14.0±2.3
	2	11.5±0.8
	8	27.7±3.7
	16	28.2±2

Cultures were set up as described in Materials and Methods with 100000 chick bone marrow cells/well, 0.35 mg/well (20%) of FCS and 1.8 mg/well of BSA. Cultures were incubated in the absence and in the presence of Sigma ovotransferrin. After 7 days, the cultures were harvested, fixed and stained by the hematoxylin–benzidine–peroxide method as described. Either large benzidine-positive colonies or groups of smaller benzidine-negative and benzidine-positive colonies were counted as burst-like erythroid colonies. Four wells were scored separately; the averages and S.D. are given.

Table 4. *Heme- and colony-stimulating activities of purified ovotransferrin*

	% benzidine+ colonies/μg	No. of colonies/μg
Ovotransferrin (as obtained from Sigma)	47.5	37.5
Trypsin-treated ovotransferrin	28.9	46.8
Hydroxylapatite fraction	32.3	48.0
Electrophoresed band	48.0	42.5
ConA-Sepharose		
fraction II	47.5	50.0
fraction III	44.0	37.5
fraction IV	45.5	45.0

Ovotransferrin was obtained from Sigma, except the ConA–Sepharose fractions which were a gift from Dr Hotta (as described by Iwase & Hotta [22]). Ovotransferrin was purified as described in Methods. Heme-stimulating activities were determined in cultures made in the presence of 10% FCS; they are expressed as the % of erythroid colonies which appeared benzidine-positive per μg of protein. Colony-stimulating activities were determined in cultures made in the absence of FCS; they are expressed as the number of erythroid colonies per μg of protein. The specific activity remained the same or slightly increased for both heme- and colony-stimulating activities. The apparent decrease of heme-stimulating activity of the 'trypsin' and 'HAP' fractions in this experiment was not confirmed (data not shown).

zidine-negative and slightly benzidine-positive at low dosages (0–2 μg of ovotransferrin). The intensity of the benzidine stain increased with the ovotransferrin concentration. No significant differences were found between the activities of iron-saturated and of iron-free ovotransferrin (data not shown). Human or rabbit serum transferrins (Sigma Chemical Co.) were not able to produce benzidine-positive chick erythroid colonies (fig. 4) nor did they stimulate chick erythroid colony formation (fig. 5).

For assaying burst-stimulating activity, cultures were set up as described in Materials and Methods in the absence and presence of several concentrations of ovotransferrin. After 7 days of incubation, the cultures were fixed and stained as described, and burst-like colonies were scored by the criteria discussed earlier. Cultures made in the absence of ovotransferrin contained an average number of 2.7 benzidine-negative bursts. Cultures made in the presence of ovotransferrin contained a maximum number of 28 benzidine-positive bursts. The number (table 3), the intensity of the benzidine stain and the size of these bursts increased with increasing amounts of ovotransferrin.

Further purification of ovotransferrin

Since it is possible that the activities demonstrated for ovotransferrin were due to trace impurities, efforts were made to study this possibility by independent further purification of commercial iron-saturated ovotransferrin (Sigma).

Iron-saturated ovotransferrin, which is

resistant to trypsin digestion [13] was digested with trypsin and the digest was separated by Sephadex G-75 chromatography. Fractions eluting in the ovotransferrin region were pooled and lyophilized (Materials and Methods).

Iron-saturated ovotransferrin fractionated by ConA–Sepharose chromatography was obtained from Dr Hotta (1977).

All the above preparations gave one single band at about 80 000 and only trace amounts of other proteins by SDS-gel electrophoresis (data not shown). Table 4 shows the heme- and colony-stimulating activities of all the above-mentioned fractions, which were not significantly different from each other and not significantly altered by the purification procedures.

DISCUSSION

By itself BSA does not have erythroid colony-stimulating activity, but in the presence of anemic chicken plasma it is able to allow the formation of small, erythroid colonies (fig. 1). Perhaps contaminants bound to BSA are responsible for this effect, because a preparation of lipid-free BSA was unable to support colony formation. It has been reported that the growth of mouse hemopoietic colonies in low serum requires BSA and lipids [6]. Maximum growth of small, erythroid colonies requires the presence of FCS even when anemic chicken plasma is added to the medium (table 1). This suggests that FCS contains a factor not present in anemic chicken plasma required for this optimal growth. Attempts to substitute the FCS requirement in mouse bone marrow erythroid cell cultures have not been completely successful, although culture conditions have been found to reduce to 1% the FCS requirement [6].

This study reports for the first time the existence of erythroid burst-like precursors (BFU-E) in chicken bone marrow. The appearance of the bursts is dependent on the presence of anemic chicken plasma. The morphology (fig. 2), the time required for formation (Materials and Methods) and the numbers of these bursts are similar to what has been reported in the mouse and in human bone marrow cultures [2, 5, 7–10].

The behavior in our in vitro system of the heme-stimulating activity during gel filtration, isoelectrofocusing, trypsin digestion (table 1), ammonium sulfate precipitation (table 1), DEAE–cellulose chromatography (table 1), HAP chromatography (table 1) and inactivation by heat at low pH (table 1) were similar to that reported for human serum transferrin or chicken ovotransferrin (table 1) [14–17]. By using published purification procedures for serum transferrin [17], a fraction could be obtained that had heme-stimulating activity and a band of 80 000 molecular weight by SDS-gel electrophoresis (data not shown). Estimations of level of transferrin in the anemic chicken plasma by SDS-gel electrophoresis and comparison of expected heme- and colony-stimulating activities on the basis of the dose response curves (figs 4, 5) for purified ovotransferrin, varied within the same orders of magnitude (data not shown). Furthermore, heme-, colony- and burst-stimulating activities from anemic chicken plasma could not be separated from each other by gel filtration or by isoelectrofocusing. All these data suggest that there is only one factor involved in the three separable effects observed for anemic chicken plasma and that this factor is probably serum transferrin.

Serum transferrin and ovotransferrin (the transferrin of egg white) seem to differ only in the carbohydrate composition [18–21]. Serum transferrin is one of the molecular

species found in the ovotransferrin when separated by ConA-Sepharose chromatography [22]. Because of the availability of highly purified ovotransferrin, experiments were continued with this material.

Ovotransferrin also appeared to have heme-stimulating activity (fig. 4), colony-stimulating activity (fig. 5) and burst-stimulating activity (table 3). Heme- and colony-stimulating activities were not removed by further purification attempts (table 4).

Samarut [23] recently reported on the preliminary purification of a chicken erythroid (in vitro) colony-stimulating factor from anemic chick serum. By gel filtration on Sephadex G-75, the peak of activity was broad and eluted near a marker at 68 000. By DEAE–cellulose, the activity was eluted at 40 mM phosphate buffer pH 6.8. Both of these properties are similar to the ones found for the heme-stimulating activity as defined in this study suggesting that Samarut's factor might be identical to our heme-stimulating factor.

The requirement for ovotransferrin (or chicken serum transferrin) by maturing red blood cells to produce benzidine-positive cells (heme-stimulating activity) is not surprising, since this iron-transporting protein is known to be required to deliver iron to those cells [24–26]. The requirement for ovotransferrin (or chicken serum transferrin) for growth (colony or burst) is also not surprising, since specific growth-promoting activities for transferrins have been reported in cell culture [27–30]. The need for ovotransferrin (or chicken serum transferrin) for growth (colony or burst) as a requirement for in vitro culture of chick erythroid cells needs further research. Specific growth-promoting activities for transferrins have been reported in cell culture for other cell types [27–30], and transferrin has been reported as a requirement (in addition to erythropoietin, BSA, FCS and Se) for mouse bone marrow erythroid cell growth [10]. No reports exist, however, on the effects of transferrin in the complete absence of FCS and/or erythropoietin in the mammalian erythrocytic cultures.

In the chick erythrocytic culture, ovotransferrin (or chicken serum transferrin) appears to be the only growth requirement in the complete absence of FCS and added specific chick erythropoietin by using the same cell concentrations and similar culture conditions to what has been used in the mouse system [3, 10].

The role of transferrin during chick erythropoiesis is not clear from our present in vitro studies. It could be that the ovotransferrin requirement appears to be the only requirement, because chicken erythropoietin and/or other chicken growth factor(s) are already present in the cell inoculum or the cells are already triggered in vivo. Although this possibility is unlikely in the case of burst formation, since it seems to require very high concentrations of erythropoietin in the mouse system [5, 6], more experimental evidence is needed to decide among this and/or other possibilities in the chick system.

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