to be any accompanying change in the synaptic organisation.

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# Partial replacement of serum by selenite, transferrin, albumin and lecithin in haemopoietic cell cultures

CULTURE media for mammalian cells usually require supplementation with serum to supply as yet undefined needs. Because the requirements are likely to be multiple, it is difficult to distinguish biologically between the effects of nonspecific 'nutritional' factors in serum and those of specific regulatory factors. Partial replacement of serum in these systems by chemically defined substances supplying the nonspecific needs would therefore represent a significant advance towards the definition of such specific factors. We have examined the role of several serum components in cultures of freshly explanted haemopoietic cells with this goal in mind.

Red-cell precursors in freshly explanted mammalian bone marrow will proliferate to form colonies if the medium contains serum and the glycoprotein hormone erythropoietin[1,2]. Similarly, colony formation by granulocyte/macrophage precursors is dependent on serum as well as a specific glycoprotein colony-stimulating factor[3]. We decreased the concentration of serum in these cultures until growth was limited. The serum concentration was made the only variable by maintaining erythropoietin or colony-stimulating factor at high and non-limiting levels. A mixture of a large number of known serum constituents was added to the cultures and restored growth. Elimination of the components one by one then established which were active and which were not. Four components—sodium selenite, transferrin, bovine serum albumin (BSA) and lecithin—accounted for all the activity of the mixture. We demonstrate here that in combination these substances replace most of the serum required for granulocyte/macrophage and erythroid colony growth and also facilitate some granulocyte/macrophage proliferation without added serum.

Bone marrow cells from the femurs of $BDF_1$ mice were plated in modified Dulbecco's medium (legend Fig. 1) made viscous with methyl cellulose[2]. Erythroid colonies of eight or more cells were identified[2] after 2 d of incubation, and granulocyte/macrophage colonies of more than 100 cells were scored after 8 d.

In these conditions, maximum numbers of erythroid colonies required the addition of 30% foetal calf serum. When the serum concentration was decreased to 1%, no colonies formed (Table 1). At this serum concentration, selenite, BSA and transferrin added individually had little effect, while in combination they stimulated the formation of large numbers of colonies. Addition of lecithin to this combination caused no further enhancement. Omission of either selenite, BSA or transferrin from the mixture resulted in a significant decrease in colony growth, while omission of serum almost completely abolished growth.

Maximum numbers of granulocyte/macrophage colonies ordinarily required the addition of 10–15% serum. When

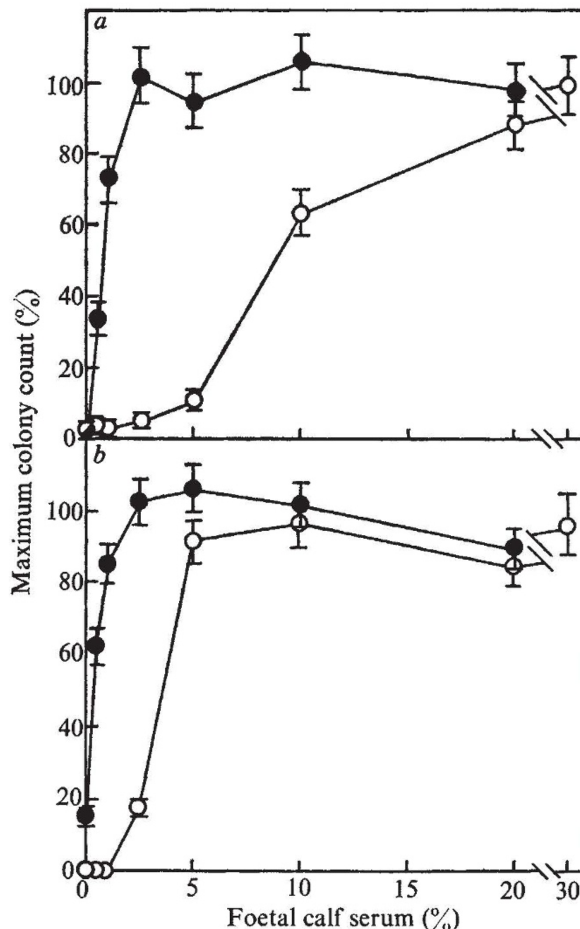


**Fig. 1** Dependence of colony formation on foetal calf serum concentration. *a*, Erythroid colonies, percentage of counts at 30% serum (240 per $10^5$ cells), 0.5 erythropoietin units per ml. ○, Nothing added; ●, supplemented with 0.75% BSA, $3.4\times10^{-6}$ M transferrin + $1.6\times10^{-6}$ M $FeCl_3$, and $10^{-7}$ M $Na_2SeO_3$. *b*, Granulocyte/macrophage colonies, percentage of counts at 30% serum (126 per $10^5$ cells), plateau amounts of colony stimulating factor. ○, Nothing added; ●, supplemented with BSA, transferrin/$FeCl_3$, $Na_2SeO_3$ and $3\times10^{-5}$ M egg lecithin. Bars indicate standard errors. Cells were cultured at $10^5$ per ml in Dulbecco's modified Eagle's MEM (Gibco H21) containing 0.8% methyl cellulose and supplemented with $10^{-4}$ M α-thioglycerol plus the following (μg ml$^{-1}$): L-alanine (25), L-asparagine.$H_2O$ (50), L-aspartic acid (30), L-cysteine (70), L-glutamic acid (75), L-proline (40), Na pyruvate (110), vitamin $B_{12}$ (0.025) and biotin (0.03). Human urinary erythropoietin was adsorbed on to benzoic acid and further purified on DEAE cellulose[4] and Sephadex G-100 (ref. 2). Colony-stimulating factor from serum-free mouse kidney cell conditioned medium[5] was partially purified by two passages over Sephadex G-150. BSA (Behringwerke, electrophoretically pure) was deionised over a mixed bed ion-exchange resin[6], charcoal (Norit A) extracted[7] (*p*H 3, 55 °C, 30 min) and chromatographed on an AcA 34 gel column to isolate the monomeric form. Human transferrin (Behringwerke) was further purified on AcA 34 and prepared as a 125× stock (38 mg lyophilised transferrin + 48 μg $FeCl_3.6H_2O$ per ml) in Dulbecco's medium. $Na_2SeO_3$ (Merck) was prepared as a 125× stock solution (2.2 μg ml$^{-1}$) in Dulbecco's medium containing 5% BSA. Egg lecithin (BDH, > 95%) was dissolved in propylene glycol (25 mg ml$^{-1}$) and diluted 10-fold to a 125× stock solution in Dulbecco's medium containing 5% BSA. Plate concentrations of propylene glycol (0.08%) were not inhibitory. Foetal calf serum was used without treatment.

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**Table 1** Effect of BSA, transferrin (Tf) and selenite (Se) on erythroid colony counts

| Addition | Colonies per $10^5$ nucleated cells |
|---|---|
| 30% Foetal calf serum (FCS) | 353±13* |
| 1% FCS | 0 |
| 1% FCS, Se | 6±3 |
| 1% FCS, BSA | 13±5 |
| 1% FCS, Tf | 4±2 |
| 1% FCS, Se, BSA, Tf | 232±9 |
| — , Se, BSA, Tf | 7±3 |
| 1% FCS, —, BSA, Tf | 117±6 |
| 1% FCS, Se, — , Tf | 42±7 |
| 1% FCS, Se, BSA, — | 67±10 |

Concentrations of BSA, Tf and Se were as in Fig 1.
*Standard error.

serum was eliminated, no colonies formed (Table 2). Selenite, BSA, transferrin or lecithin had no effect when added individually to the serum-free medium. When added in combination, however, they stimulated growth of significant numbers of colonies. Although the colonies obtained in these conditions were generally smaller than those obtained with 15% serum, many contained at least 1,000 cells and were visible to the unaided eye. Omission of either BSA or transferrin from the mixture eliminated colony formation entirely and colony numbers were also reproducibly lower in the absence of selenite or lecithin.

The serum-sparing effect of these substances is demonstrated in Fig. 1. The addition of selenite, BSA and transferrin reduced the serum required for maximum numbers of erythroid colonies from 30 to 2.5%. With the further addition of lecithin, the serum requirement for maximum granulocyte/macrophage colony growth was reduced from 10 to 2.5%. Again it was interesting that whereas erythroid colony formation was dependent on serum even in the presence of these substances, some granulocyte/macrophage colony formation was possible in the absence of serum.

**Table 2** Effect of BSA, Tf, Se and egg lecithin (lec) on granulocyte–macrophage colony counts

| Addition | Colonies per $10^5$ nucleated cells |
|---|---|
| 15% FCS, | 146±9* |
| None | 0 |
| Se | 0 |
| BSA | 0 |
| Tf | 0 |
| Lec | 0 |
| Se, BSA, Tf, lec | 57±4 |
| — , BSA, Tf, lec | 46±7 |
| Se, — , Tf, lec | 0 |
| Se, BSA, — , led | 0 |
| Se, BSA, Tf, — | 24±3 |

BSA was used in a concentration of 1.2%.
Concentrations of Tf and Se were as for Fig 1.
*Standard error.

It is possible that the activities demonstrated for the proteins BSA and transferrin were attributable to trace impurities. Efforts were made to diminish this possibility by further purification of supplied materials (legend Fig. 1). Transferrin activity was not replaced by the equivalent amount of $Fe^{3+}$ alone, nor was its activity reduced after gel permeation chromatography. Acrylamide slab gel electrophoresis[8] of purified BSA and transferrin in denaturing conditions (0.1% SDS, 0.1% $\beta$-mercaptoethanol, 2 $\mu$g of protein per slot) revealed only a single Coomassie brilliant blue-stainable band in both instances.

We anticipate that the active substances reported here will prove to have similar nonspecific, serum-replacing activity in cultures of various other cells and tissues. Selenium, present in serum at a concentration of $1.4\times10^{-7}$ M (ref. 9), has long been recognised as an essential trace element in whole animal studies[10], and McKeehan *et al.* demonstrated it to be a requirement for growth of human fibroblasts in culture[11]. The selenium-containing enzyme glutathione peroxidase is known to be present in leukocytes[12] as well as erythrocytes[13]. The requirement for transferrin by maturing red cells is not surprising, since this iron-transporting protein, present in serum at $4\times10^{-5}$ M (ref. 14), is known to be the major source of iron for these cells. It was unexpected, however, that transferrin should also prove necessary for growth of granulocyte/macrophage precursors. Taken together with the observations that transferrin has a role in the proliferative response of human lymphocytes to PHA[15], and in the growth of fibroblasts and pituitary cells in culture[16], the finding suggests a much wider requirement for this protein than has been recognised. Among numerous conceivable functions of albumin, we consider the likeliest to be that of a buffer for components of the medium present in inhibitory amounts. This suggestion predicts that the albumin requirement would diminish if the numerous defined components of the culture medium were adjusted to optimum levels.

The straightforward interpretation of our observations is that a major role of serum in culture is to provide selenium, transferrin, albumin and lipids to the medium. Our results do not, however, exclude the possibility that these entities simply substitute for other unrelated substances provided by serum. Several agents that were expected to show activity did not enhance colony formation at low serum concentrations, provided that selenite, transferrin, albumin and lecithin were present and that erythropoietin and colony-stimulating factor were maintained at non-limiting levels. Among those tested were dexamethasone ($2.5\times10^{-10}$ M), insulin ($2.5\times10^{-8}$ M), testosterone ($10^{-8}$ M), etiocholanolone and fluoxymesterone ($3\times10^{-7}$ M), prostaglandins $E_1$, $E_2$ and $F_{2}\alpha$ ($5\times10^{-9}$ M), $ZnCl_2$ ($10^{-7}$ M), and cholesterol ($3\times10^{-7}$ M).

Our observations have important technical implications. First, inclusion in culture medium of the active substances reported here greatly reduces the quantities of serum required, and in our experience has transformed several serum lots from inadequate to fully active. More important, we expect their routine inclusion in medium to simplify the search for more specific biologically active substances in conditioned media and serum by reducing the number of variables in the system.

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