Granulocyte/Macrophage-, Megakaryocyte-, Eosinophil- and Erythroid-Colony-Stimulating Factors Produced by Mouse Spleen Cells

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The formation of mature haemopoietic cells is controlled by hormones that specifically stimulate the progenitor cells of the granulocyte/macrophage, eosinophil, megakaryocyte and erythroid pathways. PWMSC medium (pokeweed-mitogen-stimulated spleen-cell-conditioned medium) is known to contain the biological activities that control the clonal proliferation of these four progenitor cells *in vitro* in semi-solid agar cultures. In this study the molecular properties of these biological activities were characterized, and all four colony-stimulating factors appear to be associated with glycoproteins. These factors were precipitated between 50 and 80%-satd. $(\text{NH}_4)_2\text{SO}_4$ and could be concentrated by ultrafiltration over a 10 000-mol.wt.-cut-off hollow-fibre membrane. Megakaryocyte- and erythroid-colony-stimulating factors were lost when the conditioned medium was dialysed at low ionic strength ($<0.03\text{ M}$). Neither asialo- nor sialo-erythropoietin was detectable in concentrated PWMSC medium or in the fractions purified from it by gel filtration on Sephadex G-150. The factors bound to concanavalin A-Sepharose were eluted with α -methyl-D-glucopyranoside (0.10 M). Analysis by gel filtration on Sephadex G-150 indicated that the apparent molecular-weight distributions of all colony-stimulating factors were identical (37 000). Treatment with neuraminidase did not alter the biological activities of any of these factors, but when the molecular weights were analysed, after neuraminidase treatment, on Sepharose CL-6B in the presence of guanidine hydrochloride (6 M) all were eluted with a mol.wt. of 24 000. Although the apparent molecular weights of the different factors were identical, charge differences were detectable by isoelectric focusing on thin-layer granulated gels. There appeared to be considerable charge heterogeneity associated with each factor, as all were focused over 2–4 pH units. The maximum activity of the granulocyte/macrophage-colony-stimulating factor on isoelectric focusing was at pH 4.8, whereas the maximum activity for the eosinophil-colony-stimulating factor was at pH 5.8. The erythroid- and megakaryocyte-colony-stimulating activities were detected in the pH ranges 4.8–5.8 and 4.6–7.1 respectively. Chromatographic differences between the granulocyte/macrophage- and eosinophil-colony-stimulating factors were also detected by hydrophobic chromatography at low ionic strength (0.15 M-NaCl) on Cibacron Blue-Sepharose and at high ionic strength [2 M- $(\text{NH}_4)_2\text{SO}_4$] on phenyl-Sepharose. Eosinophil-colony-stimulating factor bound more strongly than the other factors to both matrices. The megakaryocyte- and erythroid-colony-stimulating activities were always associated with those for granulocytes/macrophages and eosinophils. Preparations highly enriched for eosinophil-colony-stimulating factor were also obtained by DEAE-cellulose chromatography. An overall purification of 100-fold for all of the factors was achieved with the present techniques, and, although differences were observed, only granulocyte/macrophage-stimulating factors and a small proportion of the eosinophil-stimulating factors could be completely separated from the others. Our results are consistent with the existence of separable factors for granulocyte/macrophage and eosinophil stimulation, but

Abbreviations used: CS factor, colony-stimulating factor; PWMSC medium, pokeweed-mitogen-stimulated spleen-cell-conditioned medium.

the megakaryocyte- and erythroid-stimulating activities were always associated with the granulocyte/macrophage- and eosinophil-stimulating activities. Thus there may be one molecule that is able to stimulate all four colony types or four very similar molecules that are difficult to separate.

Only a limited number of experimental systems are available for the study of the control of eukaryotic cellular proliferation and differentiation (Cohen & Carpenter, 1975; Levi-Montalcini & Angeletti, 1968). Most of these systems are disadvantaged by our limited knowledge of the precursor cells from which the mature cells are formed after specific stimulation of proliferation. Haemopoiesis not only involves the proliferation of progenitor cells but the production of related but distinguishable cell types from a single multipotential progenitor cell. The pathway of proliferation and differentiation of the haemopoietic progenitor cells committed to granulocyte/macrophage formation is controlled by a specific glycoprotein hormone (Metcalf, 1977; Burgess *et al.*, 1977). The introduction of the semi-solid-agar-culture techniques for growing neutrophilic granulocyte and macrophage colonies from mouse bone-marrow cells (Bradley & Metcalf, 1966; Ichikawa *et al.*, 1966) has allowed the identification of several haemopoietic regulatory factors as well as the characterization of the progenitor cells in the granulocyte/macrophage developmental pathway. Considerable progress has been made in the characterization of the protein (colony-stimulating factor) that stimulates the proliferation and differentiation of granulocyte and macrophage progenitor cells in mice (Burgess *et al.*, 1977; Stanley & Heard, 1977). In part, the rate of proliferation and final differentiation state are controlled by the concentration of this protein, but there are some foetal CS factors that appear to commit the granulocyte/macrophage progenitor cells to macrophage differentiation (Johnson & Burgess, 1978). It is also possible to stimulate the clonal proliferation and differentiation of eosinophil (Metcalf *et al.*, 1969, 1974; Ruscetti *et al.*, 1976), megakaryocyte (Metcalf *et al.*, 1975; Nakeff & Daniels-McQueen, 1976; McLeod *et al.*, 1976) and erythroid (Stephenson *et al.*, 1971; Johnson & Metcalf, 1977) progenitor cells. The proteins responsible for the proliferation of these other haemopoietic progenitors have not been studied extensively.

When spleen cells are cultured in the presence of pokeweed mitogen the culture fluid stimulates the formation of granulocyte/macrophage, eosinophil (Metcalf *et al.*, 1974), megakaryocyte (Metcalf *et al.*, 1975), erythroid (Johnson & Metcalf, 1977) and mixed (Johnson & Metcalf, 1977) colonies. The biological activity of this culture fluid has been assumed to be due to the presence of regulators (CS factors) specific for each cell line, although it is possible that

a single multipotent haemopoietic regulator is present that stimulates not only the multipotential stem cell but all of the committed progenitor cells as well. In the report we describe the concentration, partial purification and characterization of the granulocyte/macrophage, eosinophil, megakaryocyte and erythroid regulatory factors present in PWMSC medium. Concanavalin A-Sepharose, gel filtration, isoelectric focusing and hydrophobic chromatography proved very useful techniques for the purification of all of the CS factors, and the latter two also appear to be capable of partially separating some of these haemopoietic factors from each other. The results from ion-exchange chromatography and isoelectric focusing suggest that there is considerable charge heterogeneity associated with all of the CS factors.

CS factors with different biological specificities would obviously be useful for studying the relationship between cellular proliferation and differentiation of particle haemopoietic classes (Johnson & Burgess, 1978), as well as for enumerating the frequency of the different progenitor cells. Separation of the cell-specific CS factors where they exist will provide a unique system for investigating the expression of a committed programme of differentiation and the difference between these specific programmes. The identification of a protein capable of stimulating several types of progenitor cells as well as the more specific granulocyte/macrophage- and eosinophil-stimulating factors should also allow more detailed probing of the molecular control of haemopoiesis.

Experimental Procedures

Assay cultures

The semi-solid-agar-culture system of mouse bone-marrow cells has been described in detail previously (Metcalf, 1970, 1977). Erythroid colonies were grown on the same culture system except that the bone-marrow cells were replaced by 20000 foetal liver (12 days of gestation) cells from CBAf/CaH WEHI mice, and the foetal calf and horse sera were replaced with heat-inactivated human serum (final concentration 20%, v/v). After 7 days of incubation, all discrete aggregates of 50 or more cells were scored as colonies by using an Olympus dissection microscope with indirect lighting (Metcalf & Johnson, 1979). For staining, individual colonies were removed with a fine Pasteur pipette and placed on microscope slides. After dry-

ing, cover slips were placed on the slides and the colonies stained with 0.6% orcein in 60% (v/v) acetic acid. The colony cells were examined and typed at 400× or 1000× magnification, and the colonies classified as granulocyte, macrophage or megakaryocyte according to criteria used previously (Metcalf *et al.*, 1967). Eosinophil colonies were identified by their characteristic diffuse morphology in the agar culture (Metcalf *et al.*, 1974). Erythroid colonies were identified by their characteristic red colour when illuminated with indirect light (Johnson & Metcalf, 1977). For more detailed examination, erythroid colonies were placed on microscope slides and stained with benzidine/Giemsa.

The specific activities of the CS factors were expressed as the calculated total number of colonies/10⁵ bone-marrow or foetal liver cells per mg of protein (where the protein concentration was determined by the method of Lowry *et al.*, 1951). The numbers of eosinophil, megakaryocyte and erythroid progenitors in bone marrow and foetal liver are much smaller than the numbers of granulocyte/macrophage progenitors. This small number of colonies makes the estimate of quantitative recovery for the eosinophil-, megakaryocyte- and erythroid-colony-stimulating activities less reliable (maximally stimulated cultures often yielded less than 10 eosinophil, megakaryocyte or erythroid colonies). In a particular experiment it was possible to compare the amount of each activity accurately [e.g. in different column fractions both the size of the colonies and their number accurately reflected the amount of CS factor ($\pm 20\%$)]. The maximum number of colonies of a given type varied considerably ($\pm 200\%$) when the bioassay cultures were set up on different days or in different incubators, so that all fractions from a given experiment (including the starting samples for calculation of the recovery) were assayed at the same time in the same incubator.

Erythropoietin assays

Erythropoietin and asialo-erythropoietin were assayed by using hypertransfused mice (Johnson & Metcalf, 1977) and stachyose (Sigma Chemical Co.) to block the hepatic removal of asialo-erythropoietin (Goldwasser *et al.*, 1974). Briefly, six C57BL mice (haematocrit >70%) were injected intraperitoneally twice with 0.4 ml of either 0.9% NaCl or the erythropoietin sample [2.5 units of human urinary fraction code E 6-3-15 LSL-ml; 80 units/mg from P. P. Dukes (Children's Hospital of Los Angeles, Sunset Boulevard, Los Angeles, CA, U.S.A.) on behalf of the Division of Blood Diseases and Resources of the National Heart, Lung and Blood Institute; erythropoietin units are based on the WHO International Reference Preparation]. The stachyose (0.2 ml of 1.5 mg/ml) was injected intra-

venously three times at -2, 0 and +2 h after the injection of the sample. Then 24 h after the last injection of the sample, ⁵⁹Fe (0.5 μ Ci in 0.2 ml of 0.9% NaCl) was injected. The mice were kept for 24 h before they were bled and their spleens removed to determine the uptake of ⁵⁹Fe. This assay for erythropoietin exhibited a significant response to 0.2 unit of human urinary erythropoietin (Johnson & Metcalf, 1977).

Preparation of conditioned medium

For the preparation of PWMSC medium, spleen cells (2×10^6 /ml) from C57BL mice were incubated in RPMI-1640 medium (Gibco, Grand Island, NY, U.S.A.) containing heat-inactivated (56°C for 30 min) human serum and pokeweed mitogen (5%, v/v, of a 1:15 dilution; Gibco). This medium (250 ml) was incubated in loosely capped Erlenmeyer flasks (500 ml) at 37°C in air containing CO₂ (10%). After 7 days of incubation the conditioned medium was centrifuged at 2500 g for 10 min and the supernatant fluid was removed for storage at -20°C.

PWMSC medium (1 litre) was concentrated at 50 ml by ultrafiltration (Amicon; hollow-fibre apparatus) over a Diaflo H1P10 hollow-fibre membrane. Approx. 200 ml of phosphate-buffered saline (0.02 M-sodium phosphate buffer, pH 7.3, containing 0.15 M-NaCl) was added and the solution concentrated again to 50 ml. This was repeated three times so that the final concentrated PWMSC medium (50 ml) had been equilibrated with the phosphate-buffered saline.

Neuraminidase treatment of PWMSC medium

Neuraminidase from *Clostridium perfringens* was obtained from Worthington Biochemical Corp., Freehold, NJ, U.S.A. (lot 56M404). The specific activity (0.63 unit/mg) was determined by using bovine submaxillary mucin. This neuraminidase contained less than 1 μ g of trypsin or chymotrypsin/mg as assessed by dye released from Hide Powder Azure (Calbiochem, San Diego, CA, U.S.A.) suspended in distilled water at 4 mg/ml. PWMSC medium [concentrated 20-fold by ultrafiltration after removal of the albumin by using Blue Sepharose (see below); 45 mg of protein/ml; 0.5 ml] was exchanged into 0.1 M-sodium acetate buffer, pH 5.0 (by using Sephadex G-25), was mixed with 0.25 unit of neuraminidase (1 mg/ml in water) and incubated at 37°C for 2 h. As a control an equal volume of the same solution was treated in the same way, but neuraminidase was omitted.

Fractionation Procedures

Ion exchange

Spleen-conditioned medium (15 ml) was dialysed (Union Carbide: 3 cm tubing) once against 0.01 M-

Tris/HCl (pH 7.4) followed by three changes of distilled water (2 litres) and applied to a column (1.2 cm $\times$ 4.5 cm) of DEAE-cellulose (DE-52, Whatman) equilibrated initially with 0.01 M-Tris/HCl buffer (pH 7.4), then washed exhaustively with deionized distilled water. When all of the conditioned medium had been applied and the column washed with a further 15 ml of distilled water the protein was eluted by addition of 0.1 M-NaCl followed by 0.2 M-NaCl and 0.5 M-NaCl. The flow rate of the column was 20 ml/h.

Hydrophobic chromatography

A column (1.4 cm $\times$ 8 cm) containing phenyl-Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden) was equilibrated with phosphate-buffered saline containing (NH₄)₂SO₄ (Schwartz/Mann, enzyme-grade, 2 M) and poly(ethylene glycol) 6000 (0.005%, w/v). (NH₄)₂SO₄ was added to 2 M to the concentrated PWMSC medium, and the precipitated protein was removed by centrifugation at 20000 g for 20 min; 6 ml of the supernatant fluid was applied to the phenyl-Sepharose column and washed through with starting buffer at a flow rate of 20 ml/h. The column was then eluted with a linear gradient made by mixing equal volumes of starting buffer (25 ml) and 5 mM-sodium phosphate buffer, pH 7.3, containing poly(ethylene glycol) 6000 (0.005%, w/v). Fractions (2 ml) were exchanged into phosphate-buffered saline by gel filtration (on Sephadex G-25) before assay.

A column (5 cm $\times$ 18.5 cm) containing Blue Sepharose CL-6B (Cibacron Blue-Sepharose; Pharmacia) was equilibrated with phosphate-buffered saline containing poly(ethylene glycol) 6000 (0.005%, w/v). Concentrated PWMSC medium (70 ml) was applied to the column and eluted at 40 ml/h. The bound protein was eluted by adding NaSCN (0.2 M) to the buffer. Fractions containing NaSCN were exchanged into phosphate-buffered saline by using Sephadex G-25 columns before assay.

Gel filtration

PWMSC medium (concentrated 20-fold; 20 ml) was mixed with ¹²⁵I-labelled bovine serum albumin, ¹²⁵I-labelled ovalbumin, ¹²⁵I-labelled α -chymotrypsinogen and ¹²⁵I-labelled cytochrome c, and the mixture applied to a column (2.5 cm $\times$ 80 cm) of Sephadex G-150 (Pharmacia) equilibrated with phosphate-buffered saline containing 0.005% poly(ethylene glycol) 6000. The column was run at 4°C with a pressure head of 15 cm and a flow rate of 5 ml/h. The void volume (V_0 120 ml) was determined by using Blue Dextran 2000 (Pharmacia), and the included volume (V_1 360 ml) was measured with vitamin B₁₂. Fractions (4 ml) were collected and those containing CS factor were pooled (40 ml). The

distribution coefficient K_D was calculated from the elution volumes (V_e) by using the standard equation $K_D = (V_e - V_0)/V_1$.

Concanavalin A-Sepharose

The pool from the Sephadex G-150 column which contained the CS factors (40 ml) was applied to a column (0.9 cm $\times$ 18 cm) of concanavalin A-Sepharose (Pharmacia) equilibrated with phosphate-buffered saline containing 0.005% poly(ethylene glycol) 6000. After the pooled fractions had been applied and the column washed with a further 35 ml of starting buffer, the glycoproteins were eluted with methyl- α -D-glucopyranoside (0.1 M) dissolved in the starting buffer. The column was run with a flow rate of 3–5 ml/h, and fractions (3 ml) were collected.

Sepharose CL-6B (Pharmacia) was equilibrated in phosphate-buffered saline containing guanidine hydrochloride (6 M; Sigma Chemical Co., St. Louis, MO, U.S.A.; grade 1) and packed into a glass column (2.6 cm $\times$ 37 cm). After removal of the albumin from PWMSC medium on Cibacron Blue-Sepharose (see above), the PWMSC medium concentrate (20 ml) was equilibrated with the column buffer and mixed with Blue Dextran, vitamin B₁₂, ¹²⁵I-labelled bovine serum albumin and ¹²⁵I-labelled chymotrypsinogen. The column was eluted at a flow rate of 30 ml/h at room temperature. Fractions of volume 2 ml were collected and elution positions calculated by weighing individual fractions. Molecular weights were estimated from the linear plot of log (molecular weight) versus the distribution coefficient, by using ovalbumin, horseradish peroxidase, α -chymotrypsinogen and lysozyme as standards. CS factor activity was assayed after exchanging individual fractions into phosphate-buffered saline on Sephadex G-25.

Thin-layer-gel isoelectric focusing

Isoelectric focusing was performed on a flat-bed apparatus using a thin layer of Ultradex granulated gel (LKB, Stockholm, Sweden) (Radola, 1973; Winter *et al.*, 1975). Concentrated PWMSC medium (20 ml) was desalted on a column (0.9 cm $\times$ 18 cm) of a mixed-bed ion-exchange resin [AG 501 X8 (D); Bio-Rad Laboratories, Richmond, CA, U.S.A.], diluted to 95 ml with distilled water and mixed with Ampholine solution (LKB; 40%, w/v; 2 ml of pH 5–3; 3 ml of pH 4–6). Ultradex (4 g) was added and the solution poured on to the flat bed and evaporated to 65% of the original weight. The mixture was focused at 4°C for 16 h at 2 W constant power and for 2 h at 8 W. The gel was sliced into 30 equal fractions and each was eluted with 5 ml of distilled water. After removal of the Ampholines on a column (0.9 cm $\times$ 18 cm) of the mixed-bed ion-exchange resin AG 501 X8 (D), 0.1 vol. of 0.2 M-sodium phosphate

buffer, pH 7.3, containing 1.5 M-NaCl and poly-(ethylene glycol) 6000 (0.05%, w/v) was added to each sample.

Results

Selective loss of megakaryocyte colony-stimulating factor

PWMSC medium appeared to contain at least four haemopoietic stimulating activities. Attempts at fractionation were initially preceded by dialysis of the medium against distilled water or low-ionic-strength buffers. This treatment invariably led to a selective loss of up to 75% of the factor(s) that stimulated megakaryocyte colonies to grow *in vitro*. Similar results were observed for the erythroid CS factor (S. H. M. Russell & D. Metcalf, unpublished work). The recovery of granulocyte/macrophage and eosinophil CS factors was essentially complete after the dialysis. This selective loss of megakaryocyte and erythroid CS factors was not caused by loss of megakaryocyte CS factors through the dialysis membrane, since it was never detected in the fluid surrounding the dialysis tubing. It was not lost when PWMSC medium was mixed with dialysis tubing nor on dialysis against phosphate-buffered saline.

When stored in the cold all of the CS factors were stable over a broad pH range (5–10) for 7 days. At lower pH values (<4) there was a significant loss of all colony-stimulating activities, but at higher pH values (>8) there was an apparent enhancement of the erythroid-stimulating activity.

Dose-response characteristics of different CS factors

Ultrafiltration was used to concentrate the proteins present in PWMSC medium. With a hollow-fibre H1P10 membrane a 20-fold increase in the pro-

tein concentration was achieved without any apparent loss of the CS-factor activities (Table 1). The titration of the PWMSC medium concentrate (serially 2-fold) indicated that there was sufficient megakaryocyte and eosinophil CS factors to stimulate all of the available specific progenitor cells in the bone-marrow and foetal liver cultures. (It should be noted that contaminants appeared to inhibit selectively the formation of eosinophil colonies at the highest concentrations of the unfractionated concentrate.) The concentration of granulocyte/macrophage CS factor appeared to be sufficient for maximal stimulation, even when the medium was not processed, but with erythroid CS factor no obvious plateau of erythroid-colony formation was achieved at these concentrations. The titration curves for megakaryocyte, eosinophil and granulocyte/macrophage CS factors all appeared to give linear plots of colony number against log₂ (dilution). Erythroid-colony formation was more sensitive to the dilution of the PWMSC medium concentrate, and a distinctly sigmoidal dose-response curve was observed with the higher dilutions of the concentrate. Cultures stimulated by samples containing more than one CS factor are often difficult to score: for example, the steep titration profile for erythroid CS factor may be due to the method of identification of the erythroid colonies, i.e. they are distinctly red. However, the haemoglobin content of the cells appears to decrease before the erythroid colony disappears, as detected by benzidine staining, so that at lower concentrations of erythroid CS factor some erythroid colonies will be missed. Similarly eosinophil colonies are scored by their characteristic diffuse shape (Metcalf *et al.*, 1974). When large numbers of granulocyte colonies are present the eosinophil colonies appear to become very large and diffuse and are easily missed until the concentration

Table 1. Titration of granulocyte/macrophage, eosinophil, megakaryocyte and erythroid CS factors using C57BL-mouse bone marrow and CBA-mouse foetal liver

PWMSC medium was concentrated 20-fold by ultrafiltration and 0.1 ml assayed in serial 2-fold dilutions using 1 ml cultures containing 75000 C57BL-mouse bone-marrow cells or 20000 CBA-mouse foetal liver cells (colony numbers are expressed as the mean ± s.d. for at least four separate experiments per 10⁵ cells plated).

Fold dilution of PWMSC medium concentrate	Number of colonies			
	Bone marrow			Foetal liver Erythroid
	Granulocyte/macrophage	Eosinophil	Megakaryocyte	
1	180 ± 40	5 ± 3	8 ± 4	100 ± 20
2	180 ± 40	8 ± 4	7 ± 5	80 ± 40
4	160 ± 40	8 ± 4	7 ± 3	30 ± 5
8	140 ± 40	7 ± 3	7 ± 3	15 ± 5
16	100 ± 30	5 ± 2	4 ± 2	5 ± 5
32	70 ± 20	2 ± 1	1 ± 1	0
64	20 ± 10	0	0	0
PWMSC medium	82 ± 9	3 ± 1	4 ± 4	18 ± 8

of granulocyte/macrophage CS factor is decreased sufficiently.

Apparent molecular weights

Gel filtration of the PWMSC medium concentrate on Sephadex G-150 removed most of the contaminating proteins, but all of the CS factors were eluted from the column at the same volume (Fig. 1). The apparent molecular weights of the CS factors were calculated after calibrating the column and using the linear relationship between log (molecular weight) and elution volume. The peaks for all four CS factors from PWMSC medium corresponded to an apparent mol.wt. of 37 000. After the active fractions had been pooled, a 20-fold increase in the specific activity of all of the CS factors was achieved, but no separation from each other was evident. After the activity from the Sephadex G-150 column had been pooled, the apparent recovery of all CS factors was 50–70% (Table 2). Attempts to improve the yield by the inclusion of detergents such as Triton X-100 (Burgess *et al.*, 1977) were not successful.

Lectin chromatography

The mixture of CS factors recovered after gel filtration on Sephadex G-150 was chromatographed

Fig. 1. Gel filtration of CS factors in PWMSC medium on Sephadex G-150

PWMSC medium (1 litre) was concentrated 20-fold and equilibrated with phosphate-buffered saline (pH 7.3); 20 ml was applied to a column (2.5 cm × 80 cm) of Sephadex G-150 and the column developed at 5 ml/h at 4°C. Fractions (4 ml) were collected and the absorbance (----) was monitored continuously at 280 nm. The molecular weight of CS factor was estimated from the distribution coefficient (K_D , see the text) compared with the distribution coefficients of proteins of known molecular weight: bovine serum albumin (67 000), ovalbumin (45 000) and α -chymotrypsinogen (26 000). The CS factors were analysed by using bone-marrow and foetal liver cells: ○, 10^5 granulocyte/macrophage; △, eosinophil; ▲, megakaryocyte; ●, erythroid.

Table 2. Purification of granulocyte/macrophage, eosinophil, megakaryocyte and erythroid CS factors from PWMSC medium

Procedure	Total protein (mg)	Specific activity				Yield (%)			
		Granulocyte/macrophage	Eosinophil	Megakaryocyte	Erythroid	Granulocyte/macrophage	Eosinophil	Megakaryocyte	Erythroid
Concentrated PWMSC medium (20 ml)	610	580 ± 160	1.8 ± 1.1	1.4 ± 1.2	10 ± 6	100	100	100	100
Sephadex G-150	16	16 000 ± 4100	44 ± 40	40 ± 30	200 ± 10	70	65	75	50
Concanavalin A-Sepharose	1.3	80 000 ± 9800	160 ± 70	170 ± 30	830 ± 120	30	15	25	15

The specific activities for the granulocyte/macrophage, eosinophil and megakaryocyte CS factors were calculated from 1 ml cultures containing 75 000 C57BL-mouse bone-marrow cells; the units for specific activity were chosen as colonies/ 10^5 bone-marrow cells per mg of protein. The specific activity of the erythroid CS factor was calculated from 1 ml cultures containing 20 000 CBA-mouse foetal liver cells; the units for erythroid CS factor were defined as colonies/ 10^5 foetal liver cells per mg of protein. The results are means ± s.d. for six separate experiments.

Fig. 2. Chromatography of CS factors from PWMSC medium on concanavalin A-Sepharose

The pooled fractions (40 ml) from the Sephadex G-150 column were applied to a column (0.9 cm $\times$ 18 cm) of concanavalin A-Sepharose equilibrated with phosphate-buffered saline. The column was washed with a further 30 ml of starting buffer and the bound protein eluted by adding α -methyl-D-glucopyranoside (0.1 M) to the buffer (i). The column was run at 4 ml/h and fractions (3 ml) were collected. The CS factors were analysed by using bone-marrow and foetal liver cells: $\bigcirc$, $10^{-1} \times$ granulocyte/macrophage; $\triangle$, eosinophil; $\blacktriangle$, megakaryocyte; $\bullet$, erythroid. ----, A_{280} .

Fig. 3. Ion-exchange chromatography of the CS factors in PWMSC medium on DEAE-cellulose

PWMSC medium (15 ml) was dialysed against three changes of distilled water and applied to DEAE-cellulose column (1.2 cm $\times$ 45 cm) in distilled water. The column was washed with a further 15 ml of distilled water and the protein eluted batchwise with 0.1 M-, 0.2 M- and 0.5 M-NaCl at 20 ml/h. Fractions (3 ml) were collected and analysed for $10^{-1} \times$ granulocyte/macrophage CS factor ($\blacksquare$), eosinophil CS factor ($\boxplus$) and megakaryocyte CS factor ($\blacksquare$) by using C57BL-mouse bone-marrow cells.

on concanavalin A-Sepharose. Although most of the protein passed through the column, more than 90% of all forms of CS factor bound to concanavalin A-Sepharose (Fig. 2). Neither erythroid nor megakaryocyte CS factor was detected in the break-through fractions and only a small proportion of the granulocyte/macrophage CS factor (15%) and eosinophil CS factor (3%) appeared to pass directly through the column. A block elution with α -methylglucopyranoside (0.1 M) was used in the experiment shown (Fig. 2) and all of the CS factors were eluted coincidentally. By this procedure, 90% of the protein was removed, but only 40–50% of the CS factors bound to concanavalin A-Sepharose were recovered. This resulted in a 4-fold purification of all CS factors by concanavalin A-Sepharose chromatography (Table 2). Increasing the concentration of α -methylglucopyranoside or using α -methylmannopyranoside did not appear to increase the yield for any of the CS factors.

DEAE-cellulose chromatography

Ion-exchange chromatography of the CS factors present in PWMSC medium on DEAE-cellulose in-

variably yielded very low recoveries of biological activities. In particular there was a considerable loss of megakaryocyte CS factor, and erythroid CS factor was not detectable after the chromatography. Initially the losses of biological activity occurred on dialysis of the CS factors against the low-ionic-strength buffers suitable for ion-exchange chromatography, but in some experiments sufficient activity was retained to attempt their characterization by using DEAE-cellulose (Fig. 3). After equilibration with 0.1 M-Tris/HCl (pH 7.4) and then distilled water most of the granulocyte/macrophage and eosinophil CS factor and all of the megakaryocyte CS factor appeared to bind to DEAE-cellulose; however, in the unbound pool there was a significant enrichment of eosinophil CS factor. Similar results were observed when the column and sample were equilibrated with 0.01 M-Tris/HCl, pH 7.4, although the enrichment of the eosinophil-stimulating activity was less effective. Granulocyte/macrophage, eosinophil and megakaryocyte CS factors were eluted batchwise from the column with 0.1 M-NaCl, even though 0.2 M-NaCl was required to elute most of the protein. These results suggested that some of the eosinophil CS factor had a different charge from the other CS factors, and the charge distribution associated with the bound granulocyte/macrophage, eosinophil and megakaryocyte CS factors did not appear to be identical (Fig. 3).

Isoelectric focusing

Preparative isoelectric focusing, on thin-layer

granulated gel beds, of concentrated PWMSC medium yielded further evidence that some of eosinophil and granulocyte/macrophage CS-factor activities could be partially separated from each other and from those of megakaryocyte and erythroid (Fig. 4). All of the CS factors were focused over a wide pH range (4–7.5) compared with albumin and haemoglobin (the major contaminants), which were focused over a range of only 0.5 pH unit. Most of the granulocyte/macrophage CS factor was focused at pH 4.8, although there appeared to be a minor peak near pH 5.8. Eosinophil CS factor appeared to have a higher isoelectric point (5.8), but again the distribution was very broad (4.5–8.5).

At the acidic end of the pH range (<pH 4.2) there was a significant enrichment for granulocyte/macrophage CS factor; indeed many of the fractions did not contain the other factors. The basic pH values (>pH 7.5) yielded several fractions that only stimulated eosinophil colonies. Thus it was possible to prepare, at the least, these two CS factors without other contaminating haemopoietic stimulating activities. It should be noted that the total recovery of both granulocyte/macrophage and eosinophil CS factor appeared to be only 20%, so that there is a considerable loss of total activity during isoelectric focusing. The megakaryocyte and erythroid stimulating activities were always associated with granulocyte/macrophage and

eosinophil CS factors (Fig. 4). There appears to be a slight difference in the charge distribution of erythroid and megakaryocyte CS factors, but fractions that stimulated these types of colonies invariably stimulated granulocyte/macrophage and eosinophil colonies as well. The average recovery of megakaryocyte and eosinophil CS factors was only 10%, but sufficient activity was available for a reasonable analysis of the charge distribution associated with each specificity. In part, the low recovery of the CS factors was due to the need to remove the toxic Ampholines used to generate the pH gradient (see the Fractionation Procedures section).

Hydrophobic chromatography

Hydrophobic chromatography was used initially to remove human serum albumin from the PWMSC medium concentrates. Blue Sepharose was successfully used to remove albumin, and the CS factors appeared in the breakthrough fractions (Fig. 5). The binding capacity of the Blue Sepharose was less than 5 mg of albumin/ml when the column had been equilibrated with phosphate-buffered saline. Thus a large volume of Blue Sepharose (500 ml) was needed to remove all of the albumin from 70 ml of PWMSC medium concentrated 20-fold. Although most of the protein failed to interact with the Blue Sepharose, the CS-factor peak trailed considerably and, in

Fig. 4. *Preparative thin-layer granulated gel isoelectric focusing of the CS factors in PWMSC medium*

Concentrated PWMSC medium was desalted using a mixed-bed resin [AG 501 8X(D)] diluted to 95 ml with distilled water and mixed with Ampholine solution pH 5–8 (5 ml, 40%, w/v), Ampholine solution pH 4–6 (3 ml, 40%, w/v) and Ultrodex (4 g). The slurry was poured on to a flat bed and evaporated by 35%. Initially the proteins were focused at 2 W constant power for 16 h at 4°C and finally at 8 W for a further 2 h. The positions at which bovine serum albumin and haemoglobin focused under these conditions are indicated by the horizontal bars. After removal of the Ampholines with a mixed-bed resin, $10^{-1} \times$ granulocyte/macrophage CS factor (○), eosinophil CS factor (Δ), megakaryocyte CS factor (▲) and erythroid CS factor (●) were analysed by using C57BL-mouse bone-marrow cells.

Fig. 5. *Blue Sepharose chromatography of the CS factors present in PWMSC medium*

Concentrated PWMSC medium (70 ml) was applied to a column (5 cm $\times$ 18.5 cm) and developed at 40 ml/h with phosphate-buffered saline containing poly(ethylene glycol) 6000 (0.005%, w/v). Protein bound to the column was eluted by adding NaSCN (0.2 M) to the starting buffer (i). NaSCN was removed by gel filtration before the assay of the following CS factors: ○, $10^{-1} \times$ granulocyte/macrophage; Δ, eosinophil; ▲, megakaryocyte; ●, erythroid. ———, A_{280} .

particular, the eosinophil CS factor was retarded by the matrix. Indeed many of the fractions on the trailing edge of the breakthrough activity appear to stimulate eosinophil colonies specifically (Fig. 5). Some of these fractions also appeared to contain only the granulocyte/macrophage- and eosinophil-stimulating activities, but whenever the megakaryocyte- and erythroid-stimulating activities were detected the granulocyte/macrophage- and eosinophil-stimulating activities were also present. All of the stimulating activities were detected in the thiocyanate eluate (Fig. 5). Hydrophobic forces are known to increase with the increasing ionic strength, and since there appeared to be an interaction between the eosinophil CS factor and the hydrophobic column, the effect of increasing the ionic strength with (NH₄)₂SO₄ on the elution profile was investigated. Precipitation of all of the activities occurred between 50 and 80% saturation (Table 3), and the recovery of all factors was high (>70%). Thus, for the hydrophobic chromatography of PWMSC medium on phenyl-Sepharose (NH₄)₂SO₄ (2M) was added to the concentrate, the precipitated proteins were removed by centrifugation and the supernatant fluid containing the haemopoietic regulators was applied to the column. The CS factors bound to phenyl-Sepharose under these conditions, although a significant proportion of the granulocyte/macrophage CS factor only appeared to leach from the column (Fig. 6). When the ionic strength of the eluting buffer was decreased, all four CS factors were eluted before the major protein peak. However, some fractions on the trailing edge of the peak (Fig. 6) appeared to contain eosinophil CS factor free of contamination by the other CS factors. The recovery of all activities was greater than 50%, and a 10-fold purification of granulocyte/macrophage,

megakaryocyte and erythroid CS factors was achieved. Eosinophil CS factor was more closely associated with the major protein peak, and the purification was only 4-fold. However, some frac-

Fig. 6. Phenyl-Sepharose chromatography of CS factors in PWMSC medium at high ionic strength (NH₄)₂SO₄ (2M) was added to PWMSC medium and the precipitate removed by centrifugation before application of 6 ml to a column of phenyl-Sepharose equilibrated with phosphate-buffered saline containing (NH₄)₂SO₄ (2M) and poly(ethylene glycol) 6000 (0.005%, w/v). The column was developed at 20 ml/h with starting buffer, followed by a linear gradient of starting buffer (25 ml) and limiting buffer: a 1:1 mixture of sodium phosphate buffer [5 mM, pH 7.3, containing poly(ethylene glycol) 6000, (0.005%, w/v)] and ethylene glycol (25 ml). Each fraction was exchanged into phosphate-buffered saline before the 10⁻¹ × granulocyte/macrophage CS factor (○), eosinophil CS factor (△), megakaryocyte CS factor (▲) and erythroid CS factor (●) were assayed. ----, A₂₈₀; ■, conductivity.

Table 3. (NH₄)₂SO₄ precipitation of granulocyte/macrophage, eosinophil, megakaryocyte and erythroid CS factors from PWMSC medium

The PWMSC medium was concentrated 20-fold by hollow-fibre ultrafiltration with a Diaflo H1P10 cartridge. Solid (NH₄)₂SO₄ was added to the indicated percentage of saturation and granulocyte/macrophage, eosinophil and megakaryocyte CS factors were assayed on 75000 C57BL adult mouse bone-marrow cells, and erythroid CS factor was assayed on 20000 CBA 12-day foetal liver cells. The results are expressed as the number of colonies/10⁵ cells plated per ml of PWMSC medium.

(NH ₄) ₂ SO ₄ (% satn.)	10 ⁻¹ × Number of colonies (% of initial)			
	Granulocyte/ macrophage	Eosinophil	Megakaryocyte	Erythroid
Starting material	220 (100)	12 (100)	18 (100)	120 (100)
0-30	0	0	0	0
40	22 (10)	1.6 (10)	0.4 (0)	8.0 (5)
50	32 (15)	4.0 (30)	0.8 (5)	4.0 (5)
60	58 (30)	4.8 (40)	2.8 (15)	11.0 (10)
70	94 (45)	6.4 (50)	7.2 (40)	60.0 (50)
80	49 (20)	2.4 (20)	1.2 (5)	1.0 (0)
80-100	0	0	0	0
Total recovery	273 (120)	19.2 (150)	12.4 (65)	84 (70)

tions appeared to contain eosinophil CS factor free of contamination by the other CS factors.

Anomalous gel-filtration characteristics after neuraminidase treatment

The apparent molecular weight (37 000) of all of the CS factors from PWMSC medium on Sephadex G-150 was considerably higher than that reported for granulocyte/macrophage CS factor from mouse-lung-conditioned medium (Burgess *et al.*, 1977). Studies with several other granulocyte/macrophage CS factors from heart, muscle, thymus, kidney and salivary gland (Nicola *et al.*, 1979) indicated that gel-filtration chromatography in the presence of guanidine hydrochloride (Mann & Fish, 1973) after neuraminidase treatment decreased the apparent molecular weights of all of the CS factors. Incubation with guanidine hydrochloride (6 M) did not destroy their biological activities; indeed, after overnight incubation in guanidine hydrochloride, the apparent recovery of granulocyte/macrophage-, megakaryocyte- and eosinophil-stimulating activities was 100% and that of erythroid CS factor 40–80%. Similarly, there was no apparent loss of any of the colony-stimulating activities in PWMSC medium as a result of neuraminidase treatment (Table 4). Chromatography of the CS factors on Sepharose CL-6B in the presence of guanidine hydrochloride yielded two peaks of colony-stimulating activity, one with a mol.wt. of 38 000, and other with a mol.wt. of 24 000. All of the activities were present in both peaks and there was no apparent separation of the activities from each other. After treatment with neuraminidase the higher-molecular-weight peak disappeared and all of the colony-stimulating activities were eluted coincidentally with an apparent mol.wt. of 24 000 (Fig. 7).

Fig. 7. *Sepharose CL-6B chromatography in the presence of guanidine hydrochloride of neuraminidase-treated CS factors in PWMSC medium*

CS factor concentrate (0.5 ml) from Blue Sepharose was treated with neuraminidase (0.25 unit) for 2 h at 37°C; it was then added to 6 M-guanidine hydrochloride and the sample chromatographed on a column (2.6 cm × 37 cm) of Sepharose CL-6B equilibrated with sodium phosphate buffer (0.02 M, pH 7.3) containing NaCl (0.15 M) and guanidine hydrochloride (6 M). The elution volumes of proteins of known molecular weight were used to estimate the molecular weight (24 000) of the CS factors. The elution volumes for three of the marker proteins, ovalbumin (45 000), α-chymotrypsinogen (26 000) and lysozyme (14 000), are indicated. Each fraction was exchanged into phosphate-buffered saline by using Sephadex G-25 before the granulocyte/macrophage CS factor (○), 5 × eosinophil CS factor (△), 5 × megakaryocyte CS factor (▲) and 2 × erythroid CS factor (●) were assayed on (a) bone-marrow cells and (b) foetal liver cells. — — —, A₂₈₀.

Table 4. *Effect of neuraminidase treatment on biological activities of granulocyte/macrophage, eosinophil, megakaryocyte and erythroid CS factors*

PWMSC medium (0.5 ml of a 20-fold concentrate after Blue Sepharose chromatography) was incubated with or without 0.25 unit of neuraminidase from *Clostridium perfringens* for 2 h at 37°C in 0.1 M-sodium acetate buffer, pH 5.0. Granulocyte/macrophage, eosinophil and megakaryocyte colonies were assayed on 75 000 C57BL adult mouse bone-marrow cells. Granulocyte/macrophage and erythroid colonies were also assayed on 20 000 12-day CBA foetal liver cells.

Dilution of PWMSC medium concentrate	Colonies on bone-marrow cells			Colonies on foetal-liver cells	
	Granulocyte/ macrophage	Eosinophil	Megakaryocyte	Granulocyte/ macrophage	Erythroid
— Neuraminidase					
1:10	82	9	6	74	10
1:20	68	9	5	83	12
1:40	57	5	1	61	3
+ Neuraminidase					
1:10	102	13	2	90	13
1:20	92	14	1	84	9
1:40	94	11	3	70	4

Erythroid-colony-stimulating activity has no erythropoietic activity

Although the molecular properties (i.e. molecular weight and lectin-binding characteristics) of erythroid CS factor were apparently different from those of human urinary and sheep plasma erythropoietin, the possibility that it corresponded to murine erythropoietin still existed. The biological activity of erythropoietin has been defined by its effect on the survival of erythroblasts *in vitro* and its ability to increase the incorporation of ^{59}Fe into haemoglobin *in vivo*. Untreated PWMSC medium does not contain detectable erythropoietin, nor does it inhibit the detection of erythropoietin in the hypertransfused-mouse assay (Johnson & Metcalf, 1977). Although the semi-purified erythroid CS factor from PWMSC medium did not appear to contain significant erythropoietin activity *in vivo* (Table 5), it was possible that the erythroid activity in our cultures was due to murine asialo-erythropoietin (Johnson & Metcalf, 1977), in which case pretreatment of the hypertransfused mice with stachyose should have revealed the erythropoietic activity (Goldwasser *et al.*, 1974). However, in the presence of stachyose no erythropoietic activity was detectable in the semi-purified PWMSC medium (Table 5). This assay was capable of detecting 0.2 unit of erythropoietin. The amount of semi-purified erythroid CS factor used for the assay performed *in vivo* corresponded to 20 times the amount necessary to stimulate erythroid colonies *in vitro*. Even 4 units of erythropoietin failed to stimulate erythroid colonies in our assay system. When tested *in vitro*, with foetal liver cells cultured for 24 h, erythroid CS factor from PWMSC medium failed to support the survival of mouse reticulocytes or erythroblasts (survival <40%), whereas erythropoietin (100 units/ml) appeared to

allow at least 80% of these cells to survive for the 24 h. At low cell concentration (approx. 5×10^4 cells/ml) there was an increase apparent in the total number of reticulocytes and erythroblasts when erythropoietin was present (presumably owing to the development of earlier erythroid cells in the presence of erythropoietin). PWMSC medium did not support the survival, let alone the immediate production of these cells.

Discussion*Separability of cell-line-specific CS factors*

PWMSC medium exhibits four distinct biological activities, as assessed by stimulation of the proliferation of colonies of granulocyte/macrophages, eosinophils, megakaryocytes and erythroid cells (Johnson & Metcalf, 1977). These biological activities are associated with glycoproteins (Fig. 2), which have similar chromatographic properties on gel filtration carried out in the presence of dissociating agents such as guanidine hydrochloride (Figs. 1 and 7). Ion-exchange chromatography, hydrophobic chromatography and isoelectric focusing all indicate that some of the eosinophil- and granulocyte/macrophage-stimulating activities are associated with distinct and separable factors (Figs. 3–6). However, the erythroid- and megakaryocyte-stimulating activities were always associated with granulocyte/macrophage- and eosinophil-stimulating activities, and it is not possible to distinguish whether there is a single molecule that can stimulate all four of the haemopoietic progenitor cells, or whether there are four distinct factors that have not been separated by any of the techniques reported here. Initial purification attempts were hampered by the loss of megakaryocyte and erythroid

Table 5. Assays *in vivo* for erythropoietin and asialo-erythropoietin in partially purified erythroid CS factor from PWMSC medium by using hypertransfused C57BL mice

The erythroid CS-factor sample was prepared from concentrated PWMSC medium by removal of albumin by Blue Sepharose chromatography and concentration over a Diaflo PM-10 membrane (see the text). At a dilution of 1:40 this preparation (0.1 ml) stimulated 10 erythroid colonies/ 2×10^4 CBA foetal liver cells. Six 3-month-old C57BL mice were used per group. All mice were injected on days 1, 2, 3 and 5 with packed C57BL erythrocytes, and mean haematocrits on day 7 were above 70%. Mice were injected on days 7 and 8 with 0.4 ml of test substance and on day 9 with $0.5 \mu\text{Ci}$ of ^{59}Fe in 0.2 ml of 0.9% NaCl. Mice treated with stachyose were given injections of the sugar at -2, 0 and +2 h after injection of the test substance. Then 24 h after injection of the ^{59}Fe , radioactivity was measured in 0.2 ml of whole blood and the whole spleen. Results are means $\pm$ s.d.

Sample assayed	^{59}Fe (c.p.m.)	
	Peripheral blood	Whole spleen
1. 0.9% NaCl	850 $\pm$ 360	3000 $\pm$ 250
2. Erythroid CS factor from PWMSC medium	1100 $\pm$ 500	4500 $\pm$ 500
3. Erythroid CS factor from PWMSC medium + stachyose	1200 $\pm$ 500	3800 $\pm$ 1500
4. Erythropoietin (human urinary) (1 unit)	12 600 $\pm$ 3600	28 000 $\pm$ 7000
5. 0.9% NaCl + stachyose	650 $\pm$ 150	3700 $\pm$ 800

CS factors on dialysis at low ionic strength. This phenomenon was not apparent with granulocyte/macrophage CS factors isolated from other sources (Stanley & Heard, 1977; Burgess *et al.*, 1978) unless the protein concentration was very low. Recovery of eosinophil and granulocyte/macrophage CS factors after dialysis appeared to be quantitative, but the possibility of a small proportion of these activities corresponding to a multi-potential molecule that stimulated all four progenitor cells cannot be excluded. No CS factor was detected in the fluid surrounding the dialysis tubing. No such loss occurred during ultrafiltration, and on gel filtration both megakaryocyte and erythroid CS factors had apparent mol.wts. of 37 000. It is possible that these CS factors may aggregate at low ionic strength and be lost as micro-precipitates when the samples are sterilized by subsequent Millipore filtration.

Molecular properties of the different CS factors

Gel filtration, concanavalin A-Sepharose chromatography and $(\text{NH}_4)_2\text{SO}_4$ precipitation yielded identical profiles for all of the colony-stimulating activities in PWMSC medium. By Sephadex G-150 chromatography all of the activities were found to be associated with a single molecular-weight species (within a narrow range). Similarly, when PWMSC medium was chromatographed on concanavalin A-Sepharose all of the CS factors bound and were eluted from the column with α -methyl-D-glycopyranoside. The recovery of the CS factor bound to concanavalin A-Sepharose was always less than 50%, indicating that either these glycoproteins bound with another carbohydrate specificity as well as the α -glucopyranoside or that other forces (e.g. hydrophobic) are involved in binding the CS factors to the columns. Thus the size, glucose moieties and charge [as represented by the precipitability with $(\text{NH}_4)_2\text{SO}_4$] of these CS factors are similar.

Although apparent heterogeneity with respect to molecular weight (as determined by gel filtration) has been reported to depend on the degree of purity for several granulocyte/macrophage CS factors (Sheridan & Stanley, 1971; Stanley *et al.*, 1971; Stanley & Heard, 1977), the apparent molecular weights of the CS factors present in PWMSC medium were not influenced by their degree of purification. Although a single peak of activity was observed on Sephadex G-150, under dissociating conditions on Sepharose CL-6B two peaks of activity for all of the CS factors were detected, at mol.wts. of 38 000 and 24 000. However, treatment with neuraminidase and chromatography under dissociating conditions reduced the apparent molecular weights of all of the CS factors from PWMSC medium from 37 500 to 24 000.

Relationship between erythroid CS factor and erythropoietin

It is noteworthy that erythropoietin (Miyake *et al.*, 1977) is also a glycoprotein with a molecular weight similar to that of erythroid CS factor (in the absence of dissociating agents), but erythropoietin does not appear to bind to concanavalin A-Sepharose (Iscoe *et al.*, 1973). Although human urinary erythropoietin stimulates the proliferation of mouse erythroid colonies, erythroid CS factor does not appear to stimulate ^{59}Fe incorporation *in vivo* (Johnson & Metcalf, 1977). It has been reported that asialo-erythropoietin does not act *in vivo*, but still stimulates erythroid-colony growth *in vitro* (Goldwasser *et al.*, 1974). The possibility that erythroid CS factor was an asialo form of mouse erythropoietin was investigated further by the use of stachyose *in vivo* to prevent the clearance of proteins with terminal galactose residues. Whereas Goldwasser *et al.* (1974) restored more than 30% of the erythropoietin activity of asialo-erythropoietin by treating the rats with stachyose, there was still no effect of erythroid CS factor on ^{59}Fe incorporation into peripheral blood or spleen cells *in vivo*. Even if only 5% of the erythroid CS factor had been erythropoietic *in vivo* in the presence of stachyose, our experiment should have detected the activity. Thus the assay results obtained *in vivo* indicated that there was no substantial contamination of erythroid CS factor with murine erythropoietin. Furthermore, in contrast with erythropoietin, erythroid CS factor did not appear to influence the survival of the more mature erythroid precursors (erythroblasts and reticulocytes) in liquid cultures. It has been reported that PWMSC medium contains an activity that allows the survival of burst-forming units (erythroid) in the absence of erythropoietin (Axelrad, 1978; Iscoe, 1978; Metcalf & Johnson, 1979). These burst-forming units are earlier erythroid cells and this suggests that the action of erythroid CS factor (the active erythroid component of PWMSC medium) is on an earlier erythroid precursor than the target cell for erythropoietin.

Microheterogeneity associated with CS factors

Charge differences between eosinophil CS factor in PWMSC medium and the other CS factors were detected on DEAE-cellulose. There appeared to be some heterogeneity associated with eosinophil and granulocyte/macrophage CS factors: a small proportion of each failed to bind to DEAE-cellulose even at low ionic strengths. All of the CS factors were heterogeneous when analysed by isoelectric focusing, but eosinophil CS factor in particular was enriched at the higher pH. Charge heterogeneity has been reported for other leucocyte-derived proteins produced *in vitro*, e.g. interferon (Bose *et al.*, 1976). The heterogeneity associated with interferon

appears to be due to heterogeneity in the sialic acid content of different molecules (Bose *et al.*, 1976). Other sources of granulocyte/macrophage CS factor also appear to show charge heterogeneity (Burgess *et al.*, 1978; Nicola *et al.*, 1979), owing to differences in the sialic acid content, as shown by the change in isoelectric point of granulocyte/macrophage CS factor in mouse L-cell-conditioned medium after neuraminidase treatment (Tsuneoka & Shikita, 1977; Stanley & Heard, 1977). Chromatography of the CS factors in PWMSC medium on wheat-germ agglutinin-Sepharose confirms that these CS factors are also heterogeneous with respect to *N*-acetyl-D-glucosamine content or accessibility (Burgess *et al.*, 1978). Sialic acid does occur on granulocyte/macrophage CS factor from mouse-lung-conditioned medium (Sheridan *et al.*, 1974), and the experiments with neuraminidase confirm the presence of sialic acid on at least a proportion of all of the CS factors in PWMSC medium.

The CS factors may be displaced from phenyl-Sepharose by lowering the salt concentration or by using ethylene glycol. By using Blue Sepharose, albumin can be removed from PWMSC medium at low ionic strength (Travis *et al.*, 1976) where the CS factors fail to bind. Rechromatography of the albumin-depleted PWMSC medium at higher ionic strength and subsequent gradient elution of the CS factor allowed a purification of almost 100-fold. The interactions between the CS factors other than eosinophil CS factor, and Blue and phenyl-Sepharoses only occurred at higher ionic strengths. The hydrophobic chromatography indicated that some of the eosinophil CS factor could be separated from the others even at low ionic strength.

Eosinophilic regulators

Other eosinophilic biological activities have also been reported to be present in parasite or mitogen-stimulated spleen-conditioned media (Ruscetti *et al.*, 1976; Miller & McGarry, 1976; Colley, 1973). It is not clear whether these activities are associated with eosinophil CS factor or different molecules. The molecular nature of the other factors has not yet been investigated, but it would be interesting to define further the biological properties of the eosinophil-, erythroid- and megakaryocyte-stimulating activities. Granulocyte/macrophage CS factor appears to be capable of stimulating both mature and progenitor cells in the granulocyte/macrophage pathway (Burgess & Metcalf, 1977), and it is likely that eosinophil, megakaryocyte and erythroid CS factors also stimulate their respective differentiated cells. The activities of all CS factors *in vitro* in PWMSC medium are expressed simultaneously.

Cell-line-specific CS factors act independently

Maximal growth of all colony types appears to

take place even in the presence of the other CS factors, e.g. maximal numbers of megakaryocyte colonies are stimulated even in the presence of excess concentrations of granulocyte/macrophage CS factor. Thus the actions of these stimulators appear to be independent rather than competitive. PWMSC medium is also able to stimulate single cells to proliferate and differentiate to form clones that contain at least five mature haemopoietic cell types (Johnson & Metcalf, 1977). It is not yet clear whether there is a protein that stimulates the multipotential cells to produce the committed progenitor cells or whether the multipotential cells divide stochastically to produce a range of committed progenitor cells that are then able to proliferate and differentiate in the presence of granulocyte/macrophage, megakaryocyte, eosinophil and erythroid CS factors. The purification and separation of these haemopoietic differentiation molecules from each other should aid the elucidation of the cellular mechanism by which the mixed colonies arise.

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References

- Axelrad, A. A. (1978) in *Differentiation of Normal and Neoplastic Hematopoietic Cells* (Clarkson, B., ed.), pp. 155–163, Cold Spring Harbor Laboratory, Cold Spring Harbor
- Bose, S., Gurari-Rotman, D., Ruegg, U. T., Corley, L. & Anfinson, C. B. (1976) *J. Biol. Chem.* **251**, 1659–1662
- Bradley, T. R. & Metcalf, D. (1966) *Aust. J. Exp. Biol. Med. Sci.* **44**, 287–300
- Burgess, A. W. & Metcalf, D. (1977) *J. Cell. Physiol.* **90**, 471–484
- Burgess, A. W., Camakaris, J. & Metcalf, D. (1977) *J. Biol. Chem.* **252**, 1998–2003
- Burgess, A. W., Metcalf, D. & Russell, S. (1978) in *Differentiation of Normal and Neoplastic Hematopoietic Cells* (Clarkson, B., ed.), pp. 339–357, Cold Spring Harbor Laboratory, Cold Spring Harbor
- Cohen, S. & Carpenter, G. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 1317–1321
- Colley, D. G. (1973) *J. Immunol.* **110**, 1419–1423
- Goldwasser, E., Kung, C. K.-H. & Eliason, J. (1974) *J. Biol. Chem.* **249**, 4202–4206
- Ichikawa, Y., Pluznik, D. H. & Sachs, L. (1966) *Proc. Natl. Acad. Sci. U.S.A.* **56**, 488–495
- Iscove, N. N. (1978) in *Hematopoietic Cell Differentiation* (Golde, D. W., Cline, M. J., Metcalf, D. & Fox, C. F., eds.), Academic Press, New York, in the press
- Iscove, N. N., Sieber, F. & Winterhalter, K. H. (1973) in *Hemopoiesis in Culture: 2nd Inst. Workshop* (Robinson, W. A., ed.), pp. 44–52, publication no. (NIH 74-205), Department of Health, Education and Welfare, Washington

- Johnson, G. R. & Burgess, A. W. (1978) *J. Cell Biol.* **77**, 35–47
- Johnson, G. R. & Metcalf, D. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 3879–3882
- Levi-Montalcini, R. & Angeletti, P. U. (1968) *Physiol. Rev.* **48**, 534–569
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Mann, K. G. & Fish, W. W. (1973) *Methods Enzymol.* **26**, 28–42
- McLeod, D. L., Shreeve, M. M. & Axelrad, A. A. (1976) *Nature (London)* **261**, 492–494
- Metcalf, D. (1970) *J. Cell. Physiol.* **76**, 89–100
- Metcalf, D. (1977) *Recent Res. Cancer* **61**
- Metcalf, D. & Johnson, G. R. (1979) *J. Cell. Physiol.* **99**, 159–174
- Metcalf, D., Bradley, T. R. & Robinson, W. (1967) *J. Cell. Physiol.* **69**, 93–107
- Metcalf, D., Moore, M. A. S. & Warner, N. L. (1969) *J. Natl. Cancer Inst.* **43**, 983–997
- Metcalf, D., Parker, J., Chester, H. M. & Kincade, P. W. (1974) *J. Cell. Physiol.* **84**, 275–289
- Metcalf, D., MacDonald, H. R., Odartchenko, N. & Sordat, B. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 1744–1748
- Miller, A. M. & McGarry, M. P. (1976) *Blood* **48**, 293–300
- Miyake, T., Kung, C. K.-H. & Goldwasser, E. (1977) *J. Biol. Chem.* **252**, 5558–5564
- Nakeff, A. & Daniels-McQueen, S. (1976) *Proc. Soc. Exp. Biol. Med.* **151**, 587–592
- Nicola, N. A., Burgess, A. W. & Metcalf, D. (1979) *J. Biol. Chem.* **254**, 5290–5299
- Radola, B. J. (1973) *Biochim. Biophys. Acta* **295**, 412–428
- Ruscetti, F. W., Cypess, R. H. & Chervenick, P. A. (1976) *Blood* **47**, 757–765
- Sheridan, J. W. & Stanley, E. R. (1971) *J. Cell. Physiol.* **78**, 451–459
- Sheridan, J. W., Metcalf, D. & Stanley, E. R. (1974) *J. Cell. Physiol.* **84**, 147–158
- Stanley, E. R. & Heard, P. M. (1977) *J. Biol. Chem.* **252**, 4305–4312
- Stanley, E. R., Bradley, T. R. & Summer, M. A. (1971) *J. Cell. Physiol.* **78**, 301–317
- Stephenson, J. R., Axelrad, A. A., McLeod, D. L. & Shreeve, M. M. (1971) *Proc. Natl. Acad. Sci. U.S.A.* **68**, 1542–1546
- Travis, J., Bowen, J., Tewksbury, D., Johnson, D. & Pannell, R. (1976) *Biochem. J.* **157**, 301–306
- Tsuneoka, K. & Shikita, M. (1977) *FEBS Lett.* **77**, 243–246
- Winter, A., Perlmutter, H. & Davies, H. (1975) *LKB-Produkter AB Application Note*, 198