Characterization and partial purification of a haemopoietic cell growth factor in WEHI-3 cell conditioned medium

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A myelomonocytic leukaemia cell line, WEHI-3, releases into its growth medium factors which stimulate the development of pluripotential cells, granulocyte/macrophage progenitor cells, megakaryocytic and erythroid progenitor cells. Also present is a factor which is essential for the continued proliferation *in vitro* of a variety of haemopoietic precursor cell lines of a granulocytic nature (FDC-P cells). Characterization of this growth factor has demonstrated that it is a glycoprotein of apparent M_r 25 800, in which the carbohydrate component appears to be important for activity. After several purification steps, there is an increase in specific activity of approx. 4000-fold over the starting material. At each stage of purification, the factor necessary for the proliferation of FDC-P cells 'co-purifies' with activity which stimulates the proliferation and development of normal multipotential haemopoietic cells as well as megakaryocytic, erythroid and granulocytic committed progenitor cells. This 'co-purification' occurs to the extent that the multilineage stimulating factor and the FDC-P growth factor can be eluted from the same region of sodium dodecyl sulphate/polyacrylamide gels. Thus, evidence so far, using different starting methods and purification regimes, suggests that one molecule may have multiple activities on diverse cell types.

Haemopoietic cells proliferate *in vitro* in response to specific cell-lineage-restricted stimulatory factors which have been isolated from a variety of cells and tissue (for review see Metcalf, 1981). For example, granulocyte/macrophage progenitor cells will respond to a family of glycoproteins (collectively termed granulocyte/macrophage colony-stimulating factors or granulocyte/macrophage CSFs) of various molecular weights. When cultured in soft-gel media in the presence of granulocyte/macrophage CSFs there is a clonal expansion and differentiation of the granulocyte/macrophage colony-forming cells, resulting after 7–14 days in the production of colonies containing morphologically recognizable neutrophils and/or macrophages, i.e. the factor is inducing proliferation and allowing terminal maturation to occur (Metcalf, 1981; Pluznick & Sachs, 1966; Bradley & Metcalf, 1966). In the erythroid lineage, the most primitive progenitor

cells (the erythroid burst-forming units) respond to a factor termed 'burst-promoting activity' (Iscoe & Guilbert, 1978; Wagemaker, 1978) and develop into cells which respond to the hormone erythropoetin. Subsequent haemoglobin synthesis results in the formation of recognizable red cells. Similarly, megakaryocyte progenitor cells respond to a stimulatory activity which can induce the formation of megakaryocyte colonies in a soft-gel matrix (Metcalf *et al.*, 1975; Williams *et al.*, 1979). Such systems *in vitro*, in which there is a lineage-restricted response to an appropriate stimulatory molecule, are usually characterized by a progressive loss in proliferative ability as the colony size expands and the cells undergo maturation, i.e. at the end of the 7–14 day incubation period, the colony contains only mature cells; little or no 'self-renewal' of colony-forming cells has occurred (Metcalf *et al.*, 1979).

In fact, in the myeloid compartment of the haemopoietic system it has generally been assumed that the ability to self-renew extensively is restricted to the pluripotential stem cell, the cell which is measured by its ability to form colonies in the spleens of mice which have been irradiated and

Abbreviations used: CSF, colony-stimulating factor; h.p.l.c., high-pressure liquid chromatography; SDS, sodium dodecyl sulphate.

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transfused with haemopoietic cells (Till & McCulloch, 1961). Recently, cells able to form colonies in soft-gel media have been described, where the colonies contain terminally differentiated cells of several lineages, e.g. megakaryocytes, erythroid cells and granulocytes. Such colony-forming cells have been termed CFC-Mix and analysis of the resulting colonies has demonstrated that some, albeit limited, self-renewal has occurred (Metcalf *et al.*, 1979); i.e. the mixed colonies contain CFC-Mix as well as granulocyte/macrophage colony-forming cells and erythroid burst-forming cells. Since injection of the colony cells can also lead to the formation of spleen colonies the suggestion has been made that at least some CFC-Mix correspond to pluripotential stem cells (Metcalf *et al.*, 1979; Johnson & Metcalf, 1980).

The factor(s) necessary for the growth of murine CFC-Mix is found in lectin-stimulated mouse spleen cell conditioned medium and in medium conditioned by the growth of a murine myelomonocytic leukaemic cell line, WEHI-3 (Johnson & Metcalf, 1977; Iscove *et al.*, 1982). It was found that the stimulatory activity is not present in media conditioned by the growth of many other cells (Metcalf, 1981) although in such media, high levels of the granulocyte/macrophage-restricted stimulator are present.

This is significant in the context of the work to be reported here. It has been demonstrated that cells removed from actively haemopoietic long-term bone marrow cultures would continue to proliferate in the absence of the marrow stromal cells provided that the cells were routinely sub-cultured in the presence of spleen-conditioned medium or WEHI-3-conditioned medium (Greenberger *et al.*, 1978, 1979; Dexter *et al.*, 1980). In the absence of the conditioned medium the cells die within 48 h. The cell lines obtained did not form spleen colonies or mixed colonies *in vitro* but had a granulocytic nature when cultured either in liquid culture or cloned in soft agar (Dexter *et al.*, 1980). In other words, the WEHI-3-conditioned medium or the spleen-conditioned medium is inducing (apparently indefinite) self-renewal in a committed granulocytic precursor cell. Most other conditioned media were unable to support the continued proliferation of these cells, indicating that the factor responsible was not equivalent to most forms of granulocyte-macrophage CSF (Dexter *et al.*, 1980).

Because of the importance of the haemopoietic cell growth factor and its possible relevance in understanding the mechanisms of self-renewal in normal and leukaemic cells we have attempted to purify the active material and investigate its relationship to the other stimulatory activity present in WEHI-3-conditioned medium. The results presented show that a high degree of enrichment can be

achieved and that the haemopoietic cell growth factor co-purifies with a multi-lineage stimulatory factor.

Materials and methods

Culturing of FDC-P cells

The origin of the factor-dependent cell lines (FDC-P) has been reported previously (Dexter *et al.*, 1980). FDC-P2 was derived from B6D2F₁ long-term marrow culture. Most of these studies were performed between 6 and 20 months after establishing the cloned cell line. The stock cells were routinely subcultured every 3–4 days by a 1:10 dilution into growth medium. [Fischer's medium supplemented with horse serum at a final concentration of 20% (v/v) and antibiotics as previously described (Dexter & Testa, 1976)]. The majority of cells were non-adherent, but occasional loosely attaching cells were seen. In these conditions, the cells were maintained in exponential growth. Medium conditioned by the growth of WEHI-3 cells was used as previously described to maintain the survival and growth of the FDC-P cells (Dexter *et al.*, 1980).

Assay of the factor stimulating growth and survival of FDC-P cell lines

Activity was measured in three ways.

(1) *Titration to 50% survival.* Serial dilutions of the fractions to be tested were made in Costar 24-well tissue culture plates containing 1 ml of Fischer's medium plus 20% (v/v) horse serum per well, over a range of concentration from 10% to 0.001% or less when appropriate. Exponentially growing FDC-P cells were washed free of WEHI-3-conditioned medium by centrifugation (2×), re-suspended in growth medium without conditioned medium and inoculated into the wells at 10⁵ cells/well in 1 ml. The plates were placed in a humidified gassed incubator (air/CO₂, 19:1) and were inspected on an inverted microscope after 24 and 48 h. In the absence of WEHI-3-conditioned medium factor(s) more than 95% of the cells had disintegrated after 24 h and those remaining had a granular, nonrefractive appearance. In the presence of adequate amounts of conditioned medium, cell numbers were maintained or increased and the cells were refractile and healthy-looking. Each well was scored on a four-point scale which corresponded to 0%, 30%, 60% and 100% survival as determined by Trypan Blue exclusion. This rapid assay was extremely useful when many fractions had to be tested and it proved to be more sensitive than measuring DNA synthesis or cell proliferation. When cultures were scored by two independent operators, there was close agreement on which fractions were active and where maximum activity occurred. A unit of activity was defined as the

activity in 1 ml of medium in which factor is present at a concentration giving 50% survival after 24 h.

Specific activity =

$$\frac{\text{Volume (ml) to which fraction must be diluted to give 50\% survival}}{\text{protein content } (\mu\text{g}) \text{ of fraction}}$$

(2) *DNA synthesis.* Approx. 10^5 cells were placed in Sterilin microtest plates in 0.2 ml of Fischer's medium supplemented with 10% (v/v) horse serum and the appropriate serial dilution of test material. After 24 h incubation as above, $1 \mu\text{Ci}$ of [^3H]thymidine (Amersham International) was added per well and incubation was continued for 80 min. The cells were plated onto Whatman GF/A glass fibre filters and these were washed with 5 ml of saline, 5 ml of 10% (w/v) trichloroacetic acid, 5 ml of saline and 5 ml of ethanol. They were then dried and counted in a scintillation counter. A comparison of viability and DNA synthesis as a function of WEHI-3-conditioned medium concentration is shown in Fig. 1.

(3) *Growth curves.* In some early experiments, fractions were tested by measuring their effect on cell numbers in cultures maintained for 4–6 days. Cells were counted daily in a haemocytometer and growth curves plotted. This assay is not quantitative and is too laborious for routine use. Method (1) is preferred for most purposes.

Assays for haemopoietic cell colony stimulating fraction

Granulocyte/macrophage colony-forming cells were assayed as described previously (Dexter & Testa, 1976). Briefly, marrow cells from C57B1/6 or B6D2F₁ mice were flushed into Fischer's medium and kept at 2°C. The cells were counted in a haemocytometer and, after appropriate dilutions, were added to the plating mixture to give a final concentration of $7.5 \times 10^4/\text{ml}$. Plating mixture con-

sisted of Fischer's medium (Gibco) supplemented with 20% (v/v) horse serum (Flow Laboratories), the appropriate dilution of putative stimulating material, antibiotics and a final concentration of 0.3% agar. Aliquots (1 ml) were then pipetted into 35 mm Sterilin petri dishes and the mixture was allowed to gel at room temperature. Each assay was performed in triplicate. The plates were then placed in a fully humidified incubator, gassed with air/ CO_2 (19:1) and scored 7 days later with the aid of an Olympus dissecting microscope.

On some occasions, the cells were plated in Dulbecco's Minimal Essential Medium supplemented with 20% (v/v) foetal calf serum (Gibco). On these occasions the apparent plating efficiency of the granulocyte/macrophage colony-forming cells and morphology of the colonies was similar to that seen after plating in Fischer's medium plus horse serum. A representative titration curve of granulocyte/macrophage colonies stimulated by WEHI-3-conditioned medium and partially purified fractions is shown in Fig. 2. One unit of colony-stimulating activity was defined as the minimal concentration of test material (as determined by a titration curve as in Fig. 2) which produced plateau numbers of granulocyte/macrophage colonies, counted after 7 days incubation.

Growth of cells producing erythroid or mixed erythroid colonies (i.e. colonies containing more than one haemopoietic lineage, including erythroid cells) was determined exactly as previously described (Johnson & Metcalf, 1980).

Morphology of colonies

Individual, discrete, aggregates of cells were removed from the agar plate with a micro-haemato-

Fig. 1. Growth of FDC-P2 cells for 24 h in different dilutions of serum-free WEHI-3-conditioned medium. ●, [^3H]Thymidine incorporation; O, viable cells. Values are expressed as % of control (10% conditioned medium).

Fig. 2. Granulocyte/macrophage CSF activity of serum-free WEHI-3-conditioned medium and partly purified fractions. Δ, Serum-free WEHI-3-conditioned medium; O, P-100 fraction (see also Fig. 4); ●, h.p.l.c. fraction (see also Fig. 5).

crit tube and placed on a glass slide where they were allowed to air dry. The colonies were later stained with aceto-orcein or May–Grunwald–Giemsa plus benzidine, and viewed at 800 $\times$ magnification, to determine the cell lineage. Colonies of granulocytes and macrophages were scored as mixed granulocyte/macrophage colonies if they contained 10% or more of cells of both types. Otherwise, they were scored as 'pure' granulocyte or pure macrophage. Erythroid and mixed erythroid colonies were scored together based upon their red appearance due to haemoglobin. This was confirmed with benzidine staining. Megakaryocyte colonies were scored as by Johnson & Metcalf (1978). Routinely, 40 or more individual colonies were scored.

Re-plating of colony cells

Marrow cells (10^5) were plated in 1 ml of growth medium/0.3% agar supplemented with the appropriate concentration of test material, as above, and incubated for 7 days. At this time, two plates were taken for colony counting. The contents of the third plate were then re-suspended in 9 ml of growth medium containing agar and 10% (v/v) of WEHI-3-conditioned medium. This mixture was then replated into each of ten 35 mm petri dishes (1 ml/dish) and incubated a further 7 days before scoring colony counts and morphology.

Purification of growth factor

Concentration on phenyl–Sephadex. A column of phenyl–Sephadex (20 cm $\times$ 3 cm) was equilibrated with 1.5 M-(NH₄)₂SO₄/0.1 M-Tris/HCl, pH 7.4. To 900 ml of serum-free WEHI-3-conditioned medium was added 100 ml of 1 M-Tris/HCl, pH 7.4, and 198 g of (NH₄)₂SO₄. The mixture was left for 1 h at 4°C and was then filtered through a Whatman GF/C glass fibre filter (9 cm) and applied to the phenyl–Sephadex column at a flow rate of approx. 1 ml/min at 4°C. The column was then washed with: (1) 200 ml of 1.5 M-(NH₄)₂SO₄/0.1 M-Tris/HCl, pH 7.4; (2) 200 ml of 0.75 M-(NH₄)₂SO₄/0.1 M-Tris/HCl, pH 7.4. The break-through fraction and the washes were discarded. Active factor was eluted from the column with 200 ml of 0.1 M-Tris/HCl, pH 7.4. The phenyl–Sephadex column was cleaned for further use by washing with 250 ml of propan-2-ol/water (1:4, v/v) and then with 250 ml of 1.5 M-(NH₄)₂SO₄/0.1 M-Tris/HCl.

(NH₄)₂SO₄ fractionation. To 200 ml of 0.1 M-Tris/HCl eluate, (NH₄)₂SO₄ (63 g; 50% saturation), was added with stirring. The solution was kept for 1 h at 4°C, then centrifuged for 30 min at 13 000 g. The sediment was discarded. The supernatant was adjusted to 80% saturation by the addition of a further 50 g of (NH₄)₂SO₄ and stored overnight at 4°C. It was then centrifuged at 13 000 g for 30 min. The supernatant was discarded and the sediment

was dissolved in a total of 7.0 ml of 0.1 M-ammonium acetate.

Gel filtration. A column (66 cm $\times$ 3 cm) was packed with Sephadex G-100 (particle size 40–120 μ m) previously washed and equilibrated with 0.1 M-ammonium acetate, pH 7.0 (in earlier experiments, Bio-Gel P-100 was used instead of Sephadex G-100 with similar results). The (NH₄)₂SO₄ precipitate in 7.0 ml of 0.1 M-ammonium acetate was applied to the column, which was then eluted with upward flow at a flow rate of 25 ml/h; 50 fractions (5 ml each) were collected. A_{280} was monitored with an ISCO UA 5 absorbance monitor and chart recorder. The fractions eluting immediately after the main peak of A_{280} were tested at 10%–0.001% dilution for ability to support the survival of FDC-P2 cells for 24 h. Active fractions were freeze-dried.

Reverse-phase h.p.l.c. The freeze-dried fractions from the gel filtration step were dissolved in 2.0 ml of acetonitrile/0.1% trifluoroacetic acid (1:4, v/v) adjusted to pH 2.5 with triethylamine. The solution was centrifuged for 30 min at 13 000 g to ensure that no particulate matter would enter the column. It was then loaded onto a Lichrosorb 10 RP18 column (1 cm $\times$ 25 cm). The column was eluted at 1.0 ml/min for 30 min with the loading buffer and then for 40 min with a linear gradient of 20–80% acetonitrile in 0.1% trifluoroacetic acid adjusted to pH 2.5 with triethylamine. Elution with 80% acetonitrile was continued for a further 80 min; 150 fractions (1 ml each) were collected.

To remove acetonitrile before assay, the fractions were diluted with 3 vol. of water (otherwise they would not freeze) and freeze-dried overnight. The residue was taken up in phosphate-buffered saline containing 0.1% polyethylene glycol.

Polyacrylamide-gel electrophoresis

Partly purified fractions were analysed by electrophoresis in the presence of SDS according to Laemmli (1970). Slab gels (140 mm $\times$ 150 mm $\times$ 0.75 mm) were made as follows. Running gel: water, 4 ml; 1 M-Tris/HCl (pH 8.8)/0.27% SDS, 6 ml; 40% acrylamide/1% bisacrylamide, 8 ml; tetramethylethylenediamine, 5 μ l; 1% ammonium persulphate, 2 ml. Stacking gel: water, 6.25 ml; 40% acrylamide/1% bisacrylamide, 1.25 ml; 1 M-Tris/HCl (pH 6.8)/0.8% SDS, 1.5 ml; tetramethylethylenediamine, 2 μ l; 1% ammonium persulphate, 1 ml (running gel 12.5%T, 1% bis; stacking gel 5% T, 1% bis). The upper and low tank buffers contained 14.4 g of glycine/l adjusted to pH 8.3 with Tris base, and 0.1% SDS. In some experiments upper and lower tank buffer containing 0.05 M-boric acid adjusted to pH 8.7 with Tris base and containing 0.1% SDS was used instead of glycine buffer with equivalent results.

Before loading onto the gel, protein samples were

mixed with an equal volume of solution containing glycerol (10%, w/v), SDS (2.5%), 3-mercaptopropane-1,2-diol (5%) and Tris/HCl (pH 6.8) (0.06 M). Gels were run at 20 mA per gel until the Bromophenol Blue tracking dye reached the lower edge of the gel.

Protein bands were silver-stained by the method of Sammons *et al.* (1981), but a shorter fixing and washing procedure was used. The gel was fixed in ethanol/acetic acid/water (5:1:4, by vol.) for 15 min, then washed twice for 15 min in ethanol/acetic acid/water (2:1:17, by vol.) and finally in ethanol/acetic acid/water (20:1:79, by vol.) for 15 min. Washing was with continuous gentle agitation on a rocking platform. The gel was then transferred to AgNO₃ (3 g/l) solution for 30 min. It was then rinsed for a few seconds in distilled water and developed according to Sammons *et al.* (1981) in 0.75 M-NaOH containing 90 mg of NaBH₄/l and 8 ml of 36% formaldehyde/l. Stained gels were stored in 0.75% sodium carbonate and were photographed by transmitted light with Kodak Ektachrome 400 daylight film.

Recovery of biologically active factors from SDS gels

Samples (30 µl) of concentrated fractions from gel filtration of h.p.l.c. steps were run in duplicate on slab gels together with molecular weight markers (bovine serum albumin, di-isopropylphosphofluoridate-treated chymotrypsin and cytochrome *c*). At the end of the run the gel was divided, one portion being retained for staining and the other for assay of biological activity. The distance from the tracking dye to the top of the running gel was carefully measured. One part of the gel containing the duplicate h.p.l.c. fraction was sliced horizontally in 5 mm sections which were placed in numbered tubes and shaken overnight at 4°C in 2 ml of 5% (v/v) foetal calf serum in water. The gel slices were then removed and discarded. Serum proteins plus extracted proteins were precipitated by addition of 6 ml of acetone at -40°C (Hager & Burgess, 1980). After being left for 30 min at -20°C the precipitated protein was separated by centrifugation in capped tubes for 10 min at 13 000 g and 5°C. The supernatant (containing most of the SDS) was discarded and the pellet was washed twice with a little cold acetone. The tubes were drained and left at room temperature until most of the remaining acetone had evaporated. Each fraction was then dissolved in 1 ml of phosphate-buffered saline and tested for ability to maintain survival of FDC-P2 cells.

The other part of the gel was fixed, washed and stained as described above. When silver staining was finished, the gel was measured to determine the degree of swelling or shrinking caused by the

staining process. When comparing the stained portion of the gel with the biologically assayed portion, the change in gel dimensions was taken into account.

Deglycosylation by trifluoromethanesulphonate

The method was that of Edge *et al.* (1981). Partly purified fraction from the Sephadex G-100 step (6 ml) was divided into three 2 ml portions. These were freeze-dried and 0.2 ml of anisole was added to each. Treatment of each portion was then as follows.

(A) Trifluoromethanesulphate (0.4 ml) was added and the reaction was allowed to proceed at room temperature for 40 min. Then 10 ml of diethyl ether (pre-cooled in frozen CO₂) was added, followed by 2 ml of cold 50% (v/v) aqueous pyridine. The solutions were vortex-mixed and the ether was removed with a pasteur pipette. The extraction was repeated twice more. (B) was treated the same as (A) but the temperature was kept at 4°C during the 40 min reaction period. For (C) the protein/anisole solution was kept for 40 min at room temperature, then cooled to 4°C. Trifluoromethanesulphonate (0.4 ml) (cold) was added and the mixture was extracted immediately with very cold diethyl ether as for (A) and (B).

The aqueous phases were diluted to 2 ml with water and pyridine was removed by gel filtration on Bio-Gel P-2 columns equilibrated with phosphate-buffered saline. This step was repeated as the fractions still smelt of pyridine after the first gel filtration. Active fraction was recovered in a final volume of 4.5 ml.

Desalting

Dialysis led to variable loss of activity and was therefore avoided. Ultrafiltration on Amicon UM 10 filters also resulted in incomplete recovery of activity. Amicon YM filters, however, were satisfactory. The most useful desalting procedure was to apply fractions to Bio-Gel P-2 columns (6 ml bed volume; sample applied in 2 ml and eluted in 3 ml). Ammonium acetate was removed by freeze drying.

Trypsin digestion

WEHI-3-conditioned medium (1 ml) was incubated in the presence of 0.1% (w/v) trypsin (bovine pancreatic type III; Sigma) for 30 min at 30°C in a water bath. The reaction was stopped by the addition of 0.2% (w/v) trypsin inhibitor (egg white, type II-0; Sigma) or with horse serum. The appropriate controls were performed concurrently to show that trypsin inhibitor did not affect cell growth in the assay system. When the reaction was stopped using horse serum an adjustment was made to the amount of horse serum added to the assay cultures, to ensure that optimal growth conditions were maintained. The activities of trypsin and trypsin

inhibitor were checked by a colorimetric assay using Azocol (Calbiochem).

Solubility in trichloroacetic acid

To 2 ml samples of h.p.l.c. fraction with and without carrier protein (bovine serum albumin, 400 µg/ml) was added 0.4 ml of 50% trichloroacetic acid, and to 5 ml of crude serum-free WEHI-3-conditioned medium was added 1 ml of 50% trichloroacetic acid. After being left for 30 min at 4°C, the solutions were centrifuged for 20 min at 12000g and 4°C. The supernatants were removed and extracted three times with 2 vol. of diethyl ether and the ether phase was discarded. Portions (2 ml) of the aqueous phase were exchanged into phosphate-buffered saline on Bio-Gel P-2 columns, then tested after serial dilution for ability to maintain FDC-P cell viability.

Lectin affinity chromatography

Lens culinaris lectin was prepared from lentils by the method of Hayman & Crumpton (1972). The lectin was covalently attached to aminohexyl Sepharose CL 4B by the method of Cambiaso *et al.* (1975). The beads were washed with 50 ml of 3M-ammonium thiocyanate and then extensively with 0.1M-NaHCO₃ to remove non-covalently bound protein. About 3 mg of protein was coupled per ml of wet beads. Before use with WEHI-3-conditioned medium the efficacy of the lectin-Sepharose for separating glycoproteins from non-glycosylated proteins was checked by separating a mixture of bovine serum albumin and human γ-globulin (Sigma). Albumin was eluted with phosphate-buffered saline and then globulin with 0.1M-D-methyl mannoside.

Serum-free WEHI-3-conditioned medium (22 ml) was applied to a lectin-Sepharose column (1 cm × 4 cm) and eluted as described in the legend to Fig. 7.

Results

Production of haemopoietic growth factor in complete medium and serum-free medium

WEHI-3 cells were grown for 6 days in McCoy's medium with and without 15% (v/v) foetal calf serum. The cells were removed by centrifugation and the medium tested for its ability to support the growth of FDC-P cells (Fig. 3). Serum-free conditioned medium was somewhat less active than serum-containing medium but it was clear that serum-free medium would be a suitable starting material for further purification. In other experiments (results not shown) the rate of accumulation of activity in conditioned medium was measured. It was concluded that serum-free conditioned medium was best collected 4–6 days after incubation of the WEHI-3 cells.

Purification

(1) *Hydrophobic chromatography on phenyl-Sepharose.* WEHI-3-conditioned medium (1 litre) was absorbed to a phenyl-Sepharose column and washed and eluted as described in the Materials and methods section. Aliquots (2 ml) of each fraction were desalted on Bio-Gel P-2 columns and tested for ability to maintain survival of FDC-P2 cells for 24 and 48 h. No activity was detected in the breakthrough fraction or the 0.75M-(NH₄)₂SO₄ fraction. The fraction eluted with 0.1M-Tris/HCl maintained survival at a dilution of 0.05%; the 20% propan-2-ol fraction maintained survival at 10% dilution. Thus

Fig. 3. Comparison of growth of FDC-P2 cells in medium supplemented with serum-free and serum-containing WEHI-3-conditioned medium

(a) Conditioned medium containing 15% (v/v) foetal calf serum tested at: ○, 10%; ●, 5%; △, 1%; ▲, 0.5%. (b) Serum-free conditioned medium tested at the same concentrations.

about 99% of the activity was in the Tris/HCl eluate.

(2) $(\text{NH}_4)_2\text{SO}_4$ fractionation. No activity was detected in the precipitate formed at 50% saturation; it was recovered efficiently in the precipitate which formed overnight at 80% saturation (5°C). Higher salt concentration, e.g. 90% saturation, did not offer any advantage.

(3) *Gel filtration*. A typical profile is shown in Fig. 4. A lot of the protein (as judged by A_{280}) is eliminated in the peak which emerges just before the active fractions. By comparison with the elution volumes of markers of known molecular weight (ferritin, bovine serum albumin, lactoglobulin and cytochrome c) the molecular weight of the factor was estimated at 20000–30000.

(4) *Reverse-phase h.p.l.c.* Fig. 5 shows a typical elution profile as currently obtained with a Lichrosorb 10 RP18 column. Active fractions eluted just before the main peak of u.v.-absorbing material.

In early experiments, active factor was eluted in the middle of the acetonitrile gradient, at about 40% acetonitrile, but in recent experiments it was not eluted until the gradient was complete. This increase in retention time is progressive and may be due to deterioration of the column.

(5) *Efficiency of the purification procedure*. In order to determine the efficiency of the purification procedure, ^3H -labelled conditioned medium was prepared by culturing WEHI-3 cells for 4 days in 100ml of medium containing $5\mu\text{Ci}$ of ^3H -labelled amino acid mixture/ml. The 100ml of labelled medium was added to 900ml of non-radioactive conditioned medium and this was fractionated as described above. At each stage an aliquot of the pooled active fraction was counted for radioactivity in a scintillation counter after co-precipitation with $200\mu\text{g}$ of serum albumin. Biological activity was estimated by determining the dilution that gave approx. 50% survival after 24 h. Protein content of the initial labelled conditioned medium was measured by the Lowry method and from this the radioactivity (c.p.m.) per μg of protein was determined. The protein contents of fractions at later

Fig. 4. Coincidence of activity supporting growth of FDC-P cells with activity-stimulating growth of haemopoietic progenitor cells during purification by fractionation on Bio-gel P-100 columns

Fig. 5. Coincidence of activity supporting growth of FDC-P cells with activity stimulating growth of haemopoietic progenitor cells during purification by fractionation by reverse-phase h.p.l.c.

stages of the purification (Table 3) were calculated from their radioactivity.

Stimulation of colony formation by bone marrow cells

Conditioned medium obtained from WEHI-3 cells grown in the presence of serum contained stimulatory factors for murine granulocyte/macrophage, megakaryocytic and erythroid/mixed colony forming cells (Table 1). However, a majority of the colonies counted were of pure macrophage or mixed granulocyte/macrophage cells. Serum-free WEHI-3-conditioned medium had a similar effect, although different batches tended to vary in their potency, and generally contained significantly less activity. Following the P-100 step, an equivalent number of haemopoietic colony-forming cells could be stimulated by the active fraction, but the colony morphology had altered significantly. More colonies

contained only granulocytic cells. This was even more marked following stimulation by the active fractions obtained with h.p.l.c., where 92% of the colonies were of a 'pure' granulocyte nature (i.e. there had been enrichment for a granulocyte CSF). Moreover, both the P-100 fractions and the h.p.l.c. fractions retained their ability to stimulate the megakaryocytic and erythroid/mixed colonies. This is obviously different from the effects of a highly purified CSF derived from mouse muscle-conditioned medium (data kindly supplied by Dr. D. Metcalf) where the levels of colony stimulation are reduced but where a majority of colonies also consist only of granulocytes (i.e. the material is mainly granulocyte CSF, but with an absence of megakaryocyte and erythroid/mixed CSF).

Furthermore, colonies developing in the presence of active P-100 and h.p.l.c. fraction showed a significantly greater re-plating efficiency than did colonies grown in the unfractionated WEHI-3-conditioned medium (Table 2). Also, it should be noted that the secondary colonies developing showed various cell lineages, including granulocyte, macrophage and erythroid/mixed colonies. This indicates that addition of the P-100 or h.p.l.c. fraction led to significant proliferation (self-renewal) of different progenitor cells.

Table 1. *Stimulation of haemopoietic progenitor cells by WEHI-3-conditioned-medium-derived factor(s)*

Types of colony: G, pure granulocytic; G/M, mixed granulocytic/macrophage; M, pure macrophage; Meg, megakaryocytic; E/mix, erythroid and mixed erythroid colonies.

Stimulus	Mean number of colonies/ 10 ⁵ cells	Type of colony (%)				
		G	G/M	M	Meg	E/Mix
WEHI-3-conditioned medium	169	9	40	47	1	3
P-100 fraction	202	66	24	6	2	2
H.p.l.c. fraction	209	92	1	1	2	4
DF-28*	23	96	4	0	0	0

* Highly purified granulocytic CSF derived from mouse muscle-conditioned medium (kindly supplied by Dr. D. Metcalf).

Correlations of FDC-P cell-stimulating activity with haemopoietic cell colony-stimulating factors

We attempted to determine whether the elution profiles of the factor necessary for the growth of FDC-P cells and granulocyte, megakaryocyte and erythroid/mixed CSFs from gel filtration and h.p.l.c. columns could be distinguished in any respect. Figs. 4 and 5 show that the activities were coincident; no difference in elution profiles could be seen. However, it was also apparent from an analysis of these data that significant amounts of CSF had been lost

Table 2. *Replating of colonies present in soft agar following stimulation with non-fractionated and fractionated serum-free WEHI-3-conditioned medium*

Marrow cells (10⁵) were plated in primary culture (1 ml). Whole agar colonies were resuspended in 10 ml of fresh agar medium after 7 days incubation and re-plated (1 ml/dish) in presence of 10% WEHI-3-conditioned medium. Abbreviations are as in Table 1. N.D., not determined.

Stimulating fraction	Number of primary colonies	Number of secondary colonies	Ratio primary/secondary	Type of colony (%)			
				G	G/M	M	E/Mix
WEHI-3-conditioned medium (10%)	162	3*	54:1	0	0	100	0
P-100 fraction (1%)	250	32†	7.8:1	30	40	30	2
P-100 fraction (0.1%)	80	61	1.3:1	50	30	17	2
H.p.l.c. fraction (1%)	170	154	1.1:1	75	10	15	3
H.p.l.c. fraction (0.01%)	30	7	4.3:1	N.D.	N.D.	N.D.	N.D.

* Also hundreds of macrophage clusters of <20 cells.

† High cell background.

from the original starting material. This is illustrated in Table 3, where the units of granulocyte/macrophage CSF have been extrapolated from titration curves and colony counts (Fig. 2). Since there has been a corresponding alteration produced in the colony profile (from a macrophage granulocyte/macrophage predominance in the cultures with WEHI-3-conditioned medium to a mainly granulocyte profile in the purified fractions) it seems reasonable to suggest that much of the activity lost was granulocyte/macrophage- and macrophage CSF.

Characterization of the FDC-P growth factor

Stability. In unfractionated serum-free WEHI-3-conditioned medium the FDC-P growth factor was extremely stable. Heating at 100°C for 10 min did not substantially reduce its activity, though this was reduced after 30 min at 100°C. Exposure to pH 2 or pH 10 for 30 min did not cause any loss of activity; neither did exposure to 2% SDS, or 10% mercaptoethanol (30 min at 37°C), 80% acetonitrile or 70% acetone at room temperature. Serum-containing crude conditioned medium has been stored for up to 4 months at 37°C without appreciable loss of activity. The heat stability of the purified fraction (G-100 step) was similar to that of serum-free conditioned medium (data not shown).

Solubility in trichloroacetic acid. Partly purified factor (G-100 step) was not precipitated by 10% (w/v) trichloroacetic acid after 30 min at 4°C. However, when carrier protein (400 µg/ml) was present, more than 90% of the activity co-precipitated with the carrier. Treatment of crude serum-free WEHI-3-conditioned medium with 10% (w/v) trichloroacetic acid did not result in precipitation of the factor.

Trypsin sensitivity. Incubation of WEHI-3-conditioned medium with trypsin abolished its ability to maintain proliferation of FDC-P cells. When trypsin inhibitor was added first, followed 5 min later by trypsin, no loss of activity took place. We conclude that the stimulatory factor is partly protein.

M_r . The M_r as determined by gel filtration on Bio-Gel P-100 was 20 000–30 000. A more accurate estimate of M_r was obtained by recovering the factor from gel slices after electrophoresis in the presence

Fig. 6. Recovery of activity from polyacrylamide gel after electrophoresis of h.p.l.c. fraction in Tris/HCl/glycine/SDS

Gel slices were extracted with no previous fixing or washing. Position of marker proteins is indicated: A, bovine serum albumin; B, chymotrypsin; C, cytochrome c.

Table 3. Summary of the purification procedure described in the text

Fraction	Volume (ml)	Total protein (mg)	Units of CSF*	50% survival of FDC-P cells at dilution	Total units of activity (FDC-P assay)†	Specific activity (units/µg of protein)
WEHI-3-conditioned medium	1000	80	10 000	0.5%	2×10^5	2.5
Phenyl-Sepharose	200	7.1	N.D.	0.1%	2×10^5	28
(NH ₄) ₂ SO ₄ 80% saturation	7.0	2.2	393	0.1%	7×10^5	318
Sephadex G-100	5	0.366	397	0.0002%	$2.5 \times 10^6 \pm$	6830
H.p.l.c.	0.5	0.05	750	0.0001%	5×10^5	10 000

* Determined by titration curves as detailed in the Materials and methods section.

† Activity was estimated by serial dilution ($\times 10$) to find dilution giving 50% survival of FDC-P cells after 24 h.

‡ The increase in activity on purification is probably not real. It is within the experimental error of the assay as performed (i.e. with 10-fold dilution steps).

Fig. 7. Analysis of h.p.l.c. fraction on SDS/12% polyacrylamide gels (silver stained)

(a) Marker proteins; (b) h.p.l.c. fraction equivalent to 12.5 ml of WEHI-3-conditioned medium. A, serum albumin (M_r 66000); B, chymotrypsin (M_r 25000); C, location of biological activity.

of SDS. The activity was located at a position corresponding to a range of 22000–31000. The centre of the active band was at M_r 25800 (mean of three experiments). Results of a typical experiment are shown in Fig. 6 and a silver-stained gel in Fig. 7. Though we have made several attempts to associate activity consistently with a silver-stained band, we have not yet been able to do so unequivocally. However, it was found that other haemopoietic CSFs (granulocyte, megakaryocyte and erythroid/mixed) were eluted from the gels in a position corresponding to the FDC-P growth factor. This coincidence of activity thus followed the pattern observed with G-100 and h.p.l.c. fractions.

Lectin binding. The experiment illustrated in Fig. 8 shows that the WEHI-3 factor bound to a column of immobilized *Lens culinaris* lectin and could be eluted with α -methyl mannoside, although recovery of activity was incomplete.

Importance of oligosaccharide component for activity. Treatment of glycoproteins with trifluoromethanesulphonate has been reported to strip oligosaccharides from glycoproteins without cleaving the protein core (Edge *et al.*, 1981). Table 4 shows the results of an experiment in which the activity of a partly purified fraction was tested after mixing with trifluoromethanesulphonate and allowing reaction for (a) 40 min and (b) only a few seconds, at room temperature and at 4°C. Treatment with trifluoromethanesulphonate for 40 min at room temperature abolished all activity. Activity was not lost in the control in which trifluoromethanesulphonate was present but was not given time to react.

Discussion

Cell lines which are completely dependent upon WEHI-3-conditioned medium for their sustained proliferation have been previously reported (Greenberger *et al.*, 1978, 1979; Dexter *et al.*, 1980). We have partly purified and characterized the factor(s) which promote the survival and proliferation of these cells.

Trypsin sensitivity implies that the factor is at least partly protein, and that the integrity of this protein is necessary for activity. Binding to *Lens culinaris* lectin affinity columns suggests the presence of a carbohydrate component. We conclude that the factor is a glycoprotein.

The factor is stable to heat, extremes of pH, and other denaturing agents such as SDS, organic solvents and 7M-urea. This suggests that the protein may have an open chain form, or at least that whatever tertiary structure it has is not important for activity. There is some evidence that the carbohydrate component is necessary for activity, since activity was lost on exposure to trifluoromethanesulphonate, which is believed to cleave oligosac-

Table 4. Effect of trifluoromethanesulphonate on FDC-P growth factor (G-100 fraction)

Treatment with trifluoromethanesulphonate	Fraction at dilution ...	FDC-P cell survival after 24 h (%)		
		10%	1%	0.1%
40 min at 25°C	30	—	—	—
40 min at 4°C	100	—	—	—
Less than 1 min at 4°C	100	100	100	60

Fig. 8. Lectin affinity chromatography of serum-free WEHI-3-conditioned medium (a) and growth of FDC-P cells in medium supplemented with chromatography fractions (b).

Serum-free WEHI-3-conditioned medium (22 ml) was applied to a *Lens culinaris* lectin-Sepharose 4B column (1 cm × 4 cm) equilibrated in phosphate-buffered saline containing 4 µg of bovine serum albumin/ml. The column was washed with phosphate-buffered saline (PBS) (flow rate 12 ml/h) until A_{280} fell to near baseline level (50 ml). Elution was then continued with phosphate-buffered saline containing 0.5 M- α methyl mannoside (MM) (20 ml). The α -methyl mannoside fraction was freeze-dried, dissolved in 2 ml of phosphate-buffered saline and applied to a Bio-Rad P-2 column to remove α -methyl mannoside. The eluate was diluted to 22 ml with phosphate-buffered saline before testing for growth factor activity. ○, serum-free WEHI-3-conditioned medium (10%); ▲, eluate with phosphate-buffered saline; ●, fraction eluted with α -methyl mannose (20%).

charides from glycoproteins without damaging the protein core (Edge *et al.*, 1981).

On gel filtration, the M_r was apparently 20000–30000. A more accurate estimate of M_r was made by SDS/polyacrylamide-gel electrophoresis; biologically active material was recovered from a band of mean M_r 25 800.

The purification steps we have described give an increase of specific activity of approx. 4000-fold over crude conditioned medium. The major impurity remaining seems to be serum albumin, as may be seen on SDS/polyacrylamide gels. However, five or six other bands are also present. The purified factor is therefore still some way from being homogenous. The purification procedure we have used was based to some extent on those used by Burgess *et al.* (1980) since it seemed likely that the cell-line stimulating factor would resemble CSF factor in some of its physical properties. This supposition proved to be correct in a number of respects. There is considerable similarity between the M_r 25000 factor we have studied and the M_r 25000 T19.1 granulocyte/macrophage CSF described by Burgess *et al.* (1980); thus both are heat-stable, both are resistant to SDS and mercaptoethanol, both show loss of activity on dialysis, bind partially to

lectin columns and show charge heterogeneity. However, the biological activities differ. Unlike the 'purified' WEHI-3 factor, mouse lung CSF and CSF from the murine T-cell hybrid cell line T19.1 did not stimulate erythroid, megakaryocyte or mixed cell colonies. Thus it seems that the FDC-P growth factor is physically very similar to mouse lung granulocyte/macrophage CSF and T19.1 granulocyte/macrophage CSF but is distinct from these factors in its biological activity.

The FDC-P cell lines on which the activity of purified material has been tested are derived from long-term marrow cultures and have the following characteristics: (a) they will grow continuously in the presence of the factor; in its absence the cells die; (b) they possess a range of surface markers and cytoplasmic enzymes characteristic of granulocyte/macrophage cells (Dexter *et al.*, 1980; Garland *et al.*, 1982), although the consistent finding that some cells express theta (Thy.1) antigen leaves some doubt as to their precise lineage; (c) they are non-leukaemic; (d) they can be grown, in soft agar, in the presence of the WEHI-3 factor and produce colonies which are grossly indistinguishable from those produced from normal granulocyte/macrophage progenitor cells.

These characteristics make the cells distinct from the continuous lines of basophil-like cells reported by Schrader *et al.* (1981) (which were derived from freshly isolated haemopoietic tissue) and from the lines of basophils/mast cells described by Tertian *et al.* (1981) and originating also from long-term cultures. However, our purified active material (h.p.l.c.) also stimulates the continued proliferation of basophil-like cells (T. M. Dexter, unpublished observations) and it is likely that the same, or a closely similar, factor is involved. Furthermore, Hapel *et al.* (1981) have described the production, from fresh spleen and marrow cells, of a series of cell lines which have been claimed to be lymphoid, based upon their surface phenotypes. These cell lines also respond to a factor present in WEHI-3-conditioned medium but, unlike our cells, the cell lines frequently undergo a transformation to a factor-independent growth state. It is our experience that such transformations to factor-independent growth are rare events. However, notwithstanding these differences, a sample of purified material kindly donated by Dr. Ihle (which facilitated the growth of putative lymphoid progenitor cells) also had a stimulating effect upon the FDC-P cells. Therefore, the same factor(s) may again be implicated in the continuous growth of several cell types.

In this context, it may be even more significant that the FDC-P factor has co-purified with activity having effects upon multiple haemopoietic cell lineages. In particular, each purification step (selected for greatest activity on FDC-P cells) has shown a retention of activity stimulating multipotential cells, erythroid bursts, megakaryocyte progenitor cells and granulocytic progenitor cells. Of some interest is that much of the granulocyte/macrophage and macrophage colony-stimulating activity has been lost. Furthermore, the purified material stimulates both an increase in self-renewal of myeloid progenitor cells, as well as allowing (or inducing) differentiation of the multipotential cells and subsequent maturation into the various myeloid elements to proceed. It is possible, therefore, that we are dealing with a molecule which is not only acting as a proliferative stimulus for several cell types (facilitating continuous growth in culture) but one which has multiple functions in haemopoietic cell development. In many respects our data agree with the model put forward by Iscove *et al.* (1978, 1982) who suggested that early progenitor cells may have receptors for a lineage-independent regulatory molecule. These workers, with lectin-stimulated mouse spleen cell-conditioned medium and with WEHI-3-conditioned medium, also found that activities stimulating erythroid burst forming unit, granulocyte/macrophage colony forming cells, megakaryocyte colony forming cells and mixed colonies co-purified after gel filtration, phenyl-Sephadex and

h.p.l.c. separation. Similar results have been reported by Burgess *et al.* (1980) also using spleen cell-conditioned medium.

Presently it is difficult to compare the WEHI-3 growth factor, described in this paper, with similar fractions described by other groups (Iscove *et al.*, 1978, 1982; Schrader *et al.*, 1981; Hapel *et al.*, 1981; Tertian *et al.*, 1981). For example, different starting materials and preparative procedures have been used, recovery of active material has been variable, and the apparent differences in M_r may be related to this and other problems (e.g. differences in glycosylation). However, the finding of cross-reactivity in all these systems, both in terms of stimulating material and responding cells, indicates that very similar molecules are involved.

Therefore, the purification of the WEHI-3 factor and investigations regarding its mode of action could have far-reaching implications in understanding haemopoietic cell regulation. Certainly, available evidence so far, using different starting materials and different purification regimes, all point to the same conclusion: that one molecule may have multiple activities. Whether lineage specificity is determined by minor differences in glycosylation of a common protein backbone is now being determined.

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