The Isolation and Properties of Granulocytic Colony-Stimulating Activities from Medium Conditioned by Human Peripheral Leucocytes

By GERALD B. PRICE, JOHN S. SENN, ERNEST A. McCULLOCH
and JAMES E. TILL

*Ontario Cancer Institute, and Sunnybrook Hospital, and Departments of Medical Biophysics and Medicine,
University of Toronto, Toronto, Ont. M4X1K9, Canada*

(Received 31 October 1974)

The colony-stimulating activity detected by its ability to promote colony formation by human granulopoietic progenitor cells was partially purified from medium conditioned by human peripheral leucocytes. The purification procedure utilized $(\text{NH}_4)_2\text{SO}_4$ precipitation, DEAE-cellulose chromatography, hydroxyapatite chromatography and gel filtration on Sephadex G-150 and yielded a purification of about 1000-fold. The medium from cultures of non-leukaemic cells contained three molecular species of colony-stimulating activity with approximate molecular weights of 93 000, 36 500 and 14 700. On the basis of their sensitivity to enzymes, these species of activity appeared to be proteins. In contrast, medium from cultures of leukaemic cells contained only one detectable molecular species with colony-stimulating activity, usually with an approximate molecular weight of 36 500. The results are discussed in relation to concurrent studies on the association of the different species of colony-stimulating activity with the cell surface membrane.

In the presence of appropriate stimulatory factors, granulopoietic progenitor cells will form granulocytic colonies in cell culture. The great majority of work that has been done has utilized mouse marrow as the source of granulopoietic progenitors. When colony formation by these cells is used as a bioassay for molecules with colony-stimulating activity, such molecules can be obtained from a variety of mouse tissue sources such as lung, liver, spleen and brain (see, e.g., Sheridan & Stanley, 1971). Those that have been characterized appear to be glycoproteins (Stanley & Metcalf, 1972; Austin *et al.*, 1971), although activities from different tissue sources can be heterogeneous in their properties (Sheridan & Stanley, 1971; Sheridan & Metcalf, 1973).

Somewhat different results are obtained when human, rather than mouse, granulopoietic progenitor cells are used to assay for activity. Active material stimulatory for human cells has been detected from only a few human sources, including peripheral leucocytes (Pike & Robinson, 1970; Chervenick & Boggs, 1970; Iscove *et al.*, 1971), spleen (Paran *et al.*, 1970) and embryo kidney (Brown & Carbone, 1971). These active materials detected by using human cells seem to differ from those detected by using mouse cells, in that materials active on the human cells may be inactive on the mouse cells (Price *et al.*, 1973), or vice versa (Lind *et al.*, 1974).

The purpose of the work reported here was twofold: first, to undertake a preliminary purification and characterization of colony-stimulating

activity present in medium conditioned by cultures of normal human peripheral leucocytes (Iscove *et al.*, 1971). The purification procedure used was based on that developed by Austin (1971), using mouse granulopoietic progenitors to assay for activity in leucocyte-conditioned medium. The second objective was to examine the properties of colony-stimulating activity from leukaemic peripheral leucocytes, and to compare these with the properties of activities from normal cells. Briefly, three molecular-weight species of colony-stimulating activity were observed in media conditioned by normal cells, but only one molecular-weight species was observed in the medium of leukaemic cells.

Experimental

Materials

Enzymes. Pronase was obtained from Calbiochem, Los Angeles, Calif., U.S.A. α -Chymotrypsin and neuraminidase from *Clostridium perfringens* (type VI) were from Sigma Chemical Co., St. Louis, Mo., U.S.A. Trypsin was from Worthington Biochemical Corp., Freehold, N.J., U.S.A.

Chemicals. Sephadex G-150 and G-10 were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Whatman no. 3 filter paper, Tris base (certified primary standard), sucrose (reagent grade), NaCl (reagent grade), Na_2HPO_4 (primary standard) and NaH_2PO_4 (primary standard) were purchased from Fisher Scientific Co., Fair Lawn, N.J., U.S.A.

Whatman DEAE-cellulose (DE 32) was purchased from Reeve Angel, Clifton, N.J., U.S.A. Hydroxyapatite Bio-Gel HTP was purchased from Bio-Rad Laboratories, Richmond, Calif., U.S.A. Bovine serum albumin (crystallized), cytochrome *c* from horse heart (type VI) and phenylmethanesulphonyl fluoride were purchased from Sigma Chemical Co. Methylcellulose was purchased from Dow Chemical Co., Midland, Mich., U.S.A. Bacto-agar was purchased from Difco Laboratories, Detroit, Mich., U.S.A. Foetal calf serum and alpha medium were purchased from Flow Laboratories, Rockville, Md., U.S.A.

Methods

Cell specimens. Peripheral blood was obtained from normal volunteers; bone-marrow was obtained from patients during the course of haematological investigation or in the assessment of patients with non-haematological malignancies (non-leukaemic specimens), and patients in relapse with acute and chronic leukaemias. Cell suspensions were prepared as described by Iscove *et al.* (1971). Specimens were obtained after informed consent in accordance with the regulations of and with the approval of the Human Experimentation Committee of the University of Toronto.

Conditioned medium. Leucocyte-conditioned medium was prepared from heparinized peripheral blood leucocytes of normal and leukaemic patients (see Table 1 for descriptions of patients) as described by Iscove *et al.* (1971). Briefly, leucocytes are immobilized in 0.5% (w/v) agar, 10% (v/v)

foetal calf serum and alpha medium. The agar base is overlaid with alpha medium plus 10% (v/v) foetal calf serum at 37°C in a humidified atmosphere of CO₂+air (10:90). Overlaid medium is harvested at 7 days. Such conditioned media were filter-sterilized through 0.45 µm grid membrane units (Nalgene, Rochester, N.Y., U.S.A.) and stored at 4°C.

Assay for granulocyte-colony-stimulating activity. Granulocyte-colony-stimulating activity was assayed as described by Messner *et al.* (1973). The procedure consisted of immobilizing 2×10^5 non-adherent non-leukaemic bone-marrow cells in 0.8% (w/w) methylcellulose and alpha medium supplemented with foetal calf serum. Portions of active fractions were added to the above mixture at various concentrations (v/v) to a final volume of 1 ml of culture per dish and the dishes were incubated for 15 days in a humidified atmosphere of CO₂+air (10:90). The numbers of colonies containing more than 20 cells were then counted.

Calculation of colony-stimulating activity. Two methods were used to calculate colony-stimulating activity potency per ml of material (either medium conditioned by leucocytes or fractions of partially purified medium; see below). In the first, the results were simply expressed as numbers of colonies (*N*) per ml of material, obtained by using the expression $N = C/D$, where *C* is the mean colony count in the culture dishes, determined on the linear portion of the curve relating number of granulocyte colonies to colony-stimulating activity (Messner *et al.*, 1973), and *D* is the concentration (v/v) of the test material in the cultures. Background colony counts (*B*) obtained in the same experiments were

Table 1. Data on composition of bone marrow and peripheral blood of untreated leukaemic patients

'% blasts' are given in marrow specimens used to make the initial diagnosis of leukaemia. If two values are given, the first value represents percentage of promyelocytes detected in the marrow specimen. Blasts and promyelocytes represent immature cells which occur in high proportions (greater than 5%) in the marrow of leukaemia patients not in remission or under control. Normal marrow contains less than 5% blast cells (Dacie & Lewis, 1968). Partial differentials of peripheral blood from leukaemia patients provide information about the nature of deviation from normality. For peripheral blood, 4000–11000 leucocytes/mm³, no blast cells or promyelocytes and 40–75% polymorphonuclear granulocytes are normal ranges. Polymorphonuclear granulocytes are fully developed cells of the granulocytic genus.

Patient diagnosis	Marrow (% blasts)	$10^{-3} \times$ No. of leucocytes	% blasts and promyelocytes	% polymorpho- nuclear granulocytes
1. Acute lymphocytic leukaemia	91	6.8	62	8
2. Acute lymphocytic leukaemia	99	76.6	43	6
3. Acute myeloid leukaemia	45; 55	22.2	86	3
4. Acute myeloid leukaemia	79; 16	8.4	64	11
5. Acute myeloid leukaemia	80; 4	84.8	85	10
6. Acute myeloid leukaemia	66; 10	5.5	64	10
7. Acute myelomonocytic leukaemia	—	108	94	5
8. Chronic granulocytic leukaemia-blast crisis*	29; 4	39.6	27	70
9. Chronic granulocytic leukaemia	—	353.6	8	33

* Patient with chronic granulocytic leukaemia in blast crisis was being treated with 6-mercaptopurine and prenisone.

adjusted by the same factor (D) for purposes of comparison with the values of N . The results shown in Figs. 1–5 were calculated in this way.

The second method used to estimate activity per ml was based on the net increase (I) in colony number per ml of material, obtained by using the expression $I = (C - B)/D$, where $(C - B)$ is the net increase in colony count over the background value. This procedure is necessary to obtain a valid single estimate of added colony-stimulating activity contribution to the observed colony formation. The data in Tables 2–5 were calculated in this way.

Purification and properties of granulocyte-stimulating activities. Colony-stimulating activity from medium conditioned by human leucocytes was purified by using a scheme based on that developed by Austin (1971), by using colony formation by mouse marrow granulocyte progenitors, except that the gel-filtration step with Sephadex G-150 was placed last so that any inhibitors of colony-stimulating activity (Chan & Metcalf, 1970; Chan *et al.*, 1971; Austin, 1971) also present in the medium might be removed or diminished to reveal all potential molecular-weight species of colony-stimulating activity.

Step (I). Precipitation with $(\text{NH}_4)_2\text{SO}_4$ was carried out by sequential additions of appropriate amounts of solid $(\text{NH}_4)_2\text{SO}_4$ directly to the medium at 4°C with stirring. For the experiment of Table 2, increments of 10% saturation of $(\text{NH}_4)_2\text{SO}_4$ were added to each supernatant fluid of the previous increment and precipitation was allowed to occur at 4°C for 15 min. The precipitate was redissolved in water and equilibrated against 0.01 M-Tris-HCl (pH 6.7) by dialysis. The material that precipitated between 20 and 80% saturation was redissolved with the minimum amount of water necessary and equilibrated with 0.01 M-Tris-HCl (pH 6.7) by dialysis for use in subsequent fractionation steps.

Step (II). DEAE-cellulose chromatography was performed by applying the fractions from step (I), equilibrated with 0.1 M-Tris-HCl (pH 6.7), to the top of a column (2.5 cm $\times$ 25 cm) of DE-32 DEAE-cellulose that had also been pre-equilibrated with 0.01 M-Tris-HCl (pH 6.7). A linear NaCl gradient from 0 to 1.0 M was used for elution. Salt concentrations were measured with a conductivity meter (Radiometer type CDM2e; Copenhagen, Denmark). Fractions (5 ml) were collected, equilibrated by dialysis with phosphate-buffered saline (pH 7.2) (8 g of NaCl, 12 g of KCl, 12 g of KH_2PO_4 , 1.15 g of Na_2HPO_4 , 0.132 g of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, and 0.1 g of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, to 1 litre with water), filter-sterilized with sterile filters (0.22 μm pore size), and tested for activity. For batch purification procedures an appropriate amount of pre-equilibrated DEAE-cellulose was mixed with fractions and slowly stirred for 1 h at 4°C . The fluid was removed by filtration

with a Büchner funnel and Whatman no. 3 filters, and DEAE-cellulose was then washed with 2–3 vol. of 0.01 M-Tris-HCl (pH 6.7). The protein bound to DEAE-cellulose was then eluted with 0.25 M-NaCl in 0.01 M-Tris-HCl (pH 6.7) by stirring for 1 h at 4°C . The eluate was removed by filtration and washing with additional elution buffer. The eluate was then tested for activity or equilibrated with 1 M-sodium phosphate buffer (pH 6.5).

Step (III). Hydroxyapatite chromatography was carried out by applying the fractions from step (II), equilibrated with 1 mM-sodium phosphate buffer (pH 6.5), to the top of a column (1 cm $\times$ 10 cm) of hydroxyapatite Bio-Rad HTP pre-equilibrated with 1 mM-sodium phosphate buffer (pH 6.5). A linear NaCl gradient from 0 to 0.4 M was used for elution. Fractions (5 ml) were collected, equilibrated by dialysis with phosphate-buffered saline, filter-sterilized and tested for activity. For batch purification procedures, an appropriate amount of pre-equilibrated hydroxyapatite was mixed with fractions and slowly stirred for 1 h at 4°C . The fluid was removed by filtration with a Büchner funnel and Whatman no. 3 filters, and hydroxyapatite was then washed with 2–3 vol. of 1 mM-sodium phosphate buffer (pH 6.5). The protein was then eluted with 0.08 M-NaCl in 1 mM-sodium phosphate buffer (pH 6.5) by stirring for 1 h at 4°C . The eluate was removed by filtration and washing with additional elution buffer. The eluate was then equilibrated with phosphate-buffered saline (pH 7.2) by dialysis, filter-sterilized and tested for activity.

Step (IV). Gel filtration was done on a column (2.5 cm $\times$ 40 cm) prepared from degassed Sephadex G-150 in phosphate-buffered saline (pH 7.2). Fractions were concentrated by ultrafiltration (Amicon, Lexington, Mass., U.S.A.) using a UM-10 filter designed to retain molecules with molecular weights greater than 10000. A 3 ml concentrate was loaded on the top and separated downward by using phosphate-buffered saline (pH 7.2). Fractions (5 ml) were collected, filter-sterilized and tested for activity.

Step (V). Sedimentation in sucrose density gradients utilized linear 10 ml density gradients of 5–20% (w/v) sucrose in phosphate-buffered saline (pH 7.2). A concentrate of fractions was loaded in 0.3 ml volume, with crystalline bovine serum albumin and cytochrome *c* as markers with sedimentation velocities of 4.5S and 1.9S respectively. The gradients were placed in a SW-41 swinging-bucket rotor and centrifuged at 30000 rev./min for approx. 27 h at 4°C in a Beckman model L2-50 centrifuge. Fractions (0.5 ml) were collected, immediately diluted with 2.5 ml of phosphate-buffered saline (pH 7.2), filter-sterilized and assayed for activity and marker proteins [bovine serum albumin (E_{280}) and cytochrome *c* (E_{410})].

Enzyme digestion. Portions of normal conditioned medium after step (II) (DEAE-cellulose) and step (III) (hydroxyapatite fractionation) were subjected to enzyme digestion with Pronase (45 units/mg), trypsin (190 units/mg), α -chymotrypsin (500 units/mg) and neuraminidase from *Cl. perfringens* (type VI; 1.55 units/mg) at pH 7.2 in phosphate-buffered saline. Mixtures of colony-stimulating activity and enzymes were incubated for 6 h at 37°C. At the end of 6 h, enzyme inhibitors were added; bovine serum albumin was added to the Pronase-treated group to a final concentration of 3% (w/v), and phenylmethanesulphonyl fluoride was added to the control, the trypsin- and the α -chymotrypsin-treated groups to a final concentration of 2 mM.

Protein determination. The amount of protein contained in each fraction of colony-stimulating activity was determined by the method of Lowry *et al.* (1951), with a solution of crystalline bovine serum albumin as the standard.

Results and Discussion

$(\text{NH}_4)_2\text{SO}_4$ precipitation

Austin (1971) found that precipitation of colony-stimulating activity from human leucocyte-conditioned medium occurred between 20 and 80% satd. $(\text{NH}_4)_2\text{SO}_4$, when the activity was detected by using mouse marrow cells in the assay cultures. To test for possible differences between the activities detected by using mouse marrow and human marrow cells, the experiments were repeated by using human non-adherent marrow cells as the source of granulopoietic cells used in the assay for granulocyte colony formation. The results obtained (Table 2) indicate that precipitation of activity from two media prepared from cultures of the peripheral blood cells of normal individuals occurred between 50 and 70% satd. $(\text{NH}_4)_2\text{SO}_4$. However, in the development of a procedure for the purification of colony-stimulating activity, the 20–80% satd. $(\text{NH}_4)_2\text{SO}_4$ step (step I) was retained to ensure that in subsequent studies on colony-stimulating activity derived from leukaemic patients, the possibility of loss of an abnormal activity would be minimized.

DEAE-cellulose chromatography

The colony-stimulating activity of medium conditioned by normal human leucocytes detected by using a mouse assay was previously shown (Austin, 1971) to be eluted from DEAE-cellulose by 0.25 M-NaCl. Step (I) activity detected by using human marrow cells was observed to be eluted from DEAE-cellulose at 0.17 M-NaCl (Fig. 1). The activity (step II) was eluted as a single peak. When activity after

step (III), hydroxyapatite fractionation (see below), was returned to DEAE-cellulose, no change in elution profiles was observed. The pH changed in the eluate from 6.7 at the beginning of the elution to pH 7.2 at 0.17 M-NaCl and then increased further to pH 8.0 at 1 M-NaCl, the final eluent. The batch purification procedure, using 0.25 M-NaCl to elute colony-stimulating activity (see under 'Methods') would, of course, remove all the activity remaining bound to the DEAE-cellulose. The colony-stimulating activity in medium conditioned by leucocytes from patients with untreated acute leukaemias or chronic leukaemia in relapse was also bound and eluted successfully by the batch procedure. No significant amount of activity was seen to be eluted at higher concentrations (1 and 2 M) of NaCl.

Hydroxyapatite chromatography

The colony-stimulating activity in media conditioned by normal human leucocytes detected by using human marrow cells was also found to resemble that detected by using mouse marrow cells (Austin, 1971) when subjected to chromatography on hydroxyapatite. Step (II) activities detected by using human cells were observed to be eluted from hydroxyapatite at 0.06–0.08 M-NaCl as a single peak (Fig. 2). When this activity (step III) was returned to hydroxyapatite, no significant change in elution profile was observed. However, a batch purification procedure (see under 'Methods') using 0.08 M-NaCl to elute colony-stimulating activity did not always totally remove bound activity; some variation between experiments in the concentration of NaCl needed to remove bound activity from hydroxyapatite

Table 2. $(\text{NH}_4)_2\text{SO}_4$ precipitation of granulocyte colony-stimulating activity from medium conditioned by normal leucocytes

Colony-stimulating activity was determined by titration of precipitated fractions on 2×10^5 non-adherent normal bone-marrow cells and enumeration of net colonies stimulated. A limiting concentration of each fraction was used to estimate total colony-stimulating activity. Media LCM-1 and LCM-2 were two separate culture media conditioned by normal peripheral leucocytes.

% satd. $(\text{NH}_4)_2\text{SO}_4$	% of activity precipitated	
	Medium LCM-1	Medium LCM-2
30	0	0
40	0	0
50	0	7
60	25	34
70	44	25
80+	0	0
Total recovery (%)	69	66

Fig. 1. Elution of colony-stimulating activity (●) derived from conditioned medium of peripheral leucocytes from normal individuals after $(\text{NH}_4)_2\text{SO}_4$ precipitation (step I) from a DEAE-cellulose column by NaCl (----) Protein concentration (□) of each 5 ml fraction and background colony number (○) are also shown.

Fig. 2. Elution of colony-stimulating activity (●) derived from conditioned medium of peripheral leucocytes from normal individuals after DEAE-cellulose fractionation (step II) from a hydroxyapatite column by NaCl (----) Protein concentration (□) in each 5 ml fraction and background colony number (○) are also shown.

has been observed. The activity in conditioned medium derived from leucocytes of patients with untreated acute leukaemias or chronic leukaemias in relapse was also bound and eluted from hydroxyapatite with similar results.

Sephadex G-150 chromatography

To obtain a further purification of colony-stimulating activity, the material (step III) obtained after hydroxyapatite adsorption and elution was concentrated by ultrafiltration and separated on Sephadex

Fig. 3. Fractionation of colony-stimulating activity (●) from conditioned medium of peripheral leucocytes from normal individuals (a) and from that of a leukaemia patient (b) after hydroxyapatite fractionation (step III) of Sephadex G-150 with phosphate-buffered saline

Fractions (5 ml) were collected. The background colony number (○) is also shown. V_0 , Void volume; V_t , total volume.

G-150. In each of six different preparations of conditioned medium from normal human leucocytes, a separation into at least three apparent molecular-weight species was observed (Fig. 3a). The estimated molecular weights were greater than 100000, about 35000 and approx. 15000.

Different results were obtained when material from leukaemic patients was examined. Step (III) colony-stimulating activity in conditioned medium prepared from peripheral leucocytes of seven patients with untreated acute leukaemia and two patients with chronic leukaemia appeared to contain only one detectable molecular species (Fig. 3b). Except for material from one patient with acute leukaemia (possessing only the highest-molecular-weight species), the activity of media derived from peripheral leucocytes of untreated and relapsed leukaemic patients showed a molecular weight of approx. 35000. The material eluted by 2M-NaCl after elution of DEAE-cellulose with 0.25M-NaCl, or after elution of hydroxyapatite with 0.08M-NaCl, or not bound by hydroxyapatite, was checked to ascertain if the species of other molecular weights were present in these fractions; only the single molecular-weight species distinguished in step (III) by

Fig. 4. Sucrose-density-gradient sedimentation of three fractions of colony-stimulating activity (●) from conditioned medium of peripheral leucocytes from normal individuals after gel elution on Sephadex G-150

(a) Fraction E_1 ; (b) fraction E_2 ; (c) fraction E_3 . Bovine serum albumin (E_{280} , □) and cytochrome c (E_{410} , △) were used as sedimentation standards of 4.5S and 1.9S respectively. The background colony number (○) is also shown.

gel filtration on Sephadex G-150 was observed in any of the fractions containing residual activity.

Finally, it should be noted that Sephadex G-150 gel filtration of crude concentrates does not reveal all molecular-weight species of colony-stimulating activity. Purification is apparently required to remove inhibitors of colony-stimulating activity (Chan & Metcalf, 1970; Chan *et al.*, 1971; Austin, 1971), so that all molecular species can be detected. If such inhibitors were co-purified with the leukaemic species of colony-stimulating activity and not with the normal species, then the apparent deficiency in leukaemic species could be explained. This explanation seems unlikely, but cannot be ruled out.

Sedimentation in linear sucrose density gradients

To obtain additional information about the molecular weights of the three apparent molecular-weight species seen after chromatography of normal activity on Sephadex G-150, pooled material from each of three regions of activity (Fig. 3a) after gel filtration (step IV) was sedimented in linear sucrose gradients, by the method of Martin & Ames (1961), with bovine serum albumin and cytochrome c as markers of 4.5 and 1.9S respectively. The results are shown in Fig. 4; the estimated sedimentation coeffi-

cients for the activities of one normal medium were approx. 5.8, 3.8 and 1.9S. In a series of such experiments, with three different normal media, the range of sedimentation coefficients was 5.3–5.8S, 3.8S (similar in three experiments) and 1.9–2.4S for the three species. By using the equation $S_1/S_2 = (\text{mol.wt.}_1/\text{mol.wt.}_2)^{2/3}$ (bovine serum albumin, 4.5S and 67000; cytochrome *c*, 1.9S and 12900), we therefore would tentatively assign molecular weights of 93000 ± 3100 , 36500 and 14700 ± 1500 (mean $\pm$ s.e.m.) respectively (Siegel & Monty, 1966).

Comparison of normal and leukaemic material again revealed a difference. In Fig. 5, an example of the sedimentation of colony-stimulating activity (step III) from medium conditioned by peripheral leucocytes from a single untreated patient with acute leukaemia is shown. Only one molecular-weight species, sedimenting at approx. 3.5S, was observed, in accordance with the single species seen by gel filtration on Sephadex G-150.

Yields and degree of purification of colony-stimulating activity

When the batch purification procedure outlined above (see under 'Methods') was applied to normal media, the overall yields ranged from 6 to 30% in different experiments, and the colony-stimulating activity was purified in most cases by about 1000-fold. The results for two representative purifications are shown in Table 3. The major part of purification was achieved by the hydroxyapatite-adsorption step.

Preparations of conditioned medium derived from untreated and relapsed leukaemic patients often contain as little as one-tenth of the activity of normal media (Messner *et al.*, 1973); consequently, it was

Fig. 5. Sucrose-density-gradient sedimentation of colony-stimulating activity (●) from conditioned medium of peripheral leucocytes of a leukaemia patient after hydroxyapatite fractionation (step III)

Bovine serum albumin (E_{280} , □) and cytochrome *c* (E_{410} , Δ) were used as sedimentation standards of 4.5S and 1.9S respectively. The background colony number (○) is also shown.

Table 3. Purification of colony-stimulating activity from media conditioned by human peripheral leucocytes

For details see under 'Methods'. Expts. 1 and 2 are purification results of two separate media conditioned by normal peripheral leucocytes.

	Step	(I) (NH ₄) ₂ SO ₄ (20–80% satn.) fractionation	(II) DEAE-cellulose chromatography (0–0.25 M-NaCl)	(III) Hydroxyapatite chromatography (0–0.08 M-NaCl)	Sephadex G-150 chromatography			
					Fraction E ₁	Fraction E ₂	Fraction E ₃	Total*
Volume (ml)	Expt.	Crude						
	1	2600	2270	290	305	150	150	510
	2	1050	505	340	250	56	56	192
Protein (μg/ml)	1	5000	4900	7700	<5	<5	5	<5
	2	5500	5500	5200	<10	<5	5	<5
10 ⁻⁶ × Total activity (net colonies/fraction)	1	10.1	3.4	1.4	>0.64	0.064	0.68	2.5
	2	3.0	2.1	1.4	1.2	0.056	0.14	0.38
10 ⁻³ × Specific activity	1	0.77	0.31	0.63	>420	>110	>910	>980
(net colonies/mg of protein)	2	0.52	0.76	0.79	>480	>140	>500	>400
Recovery (%)	1	100	34	14	—	—	—	29
	2	100	68	45	—	—	—	13

* Activities from pools of fractions shown to contain activity after gel filtration of Sephadex G-150 in the three molecular-weight regions. (Fraction E₁, 100000 mol.wt.; fraction E₂, 35000 mol.wt.; fraction E₃, 15000 mol.wt.)

Table 4. *Sensitivity of colony-stimulating activity to enzyme digestion*

'Recovery' is expressed as net number of colonies over background values, relative to the control group. Phenylmethylsulphonyl fluoride was added to give a final concentration of 2 mM. Bovine serum albumin was added to give a final concentration of 3% (w/v). Neither inhibitor has been observed to inhibit granulocyte-colony formation at these concentrations. Group A refers to a medium after step (II), DEAE-cellulose fractionation. Group B refers to a medium after step (III), hydroxyapatite fractionation. Media LCM-1 and LCM-2 were two separate batches of medium conditioned by normal peripheral leucocytes. For group A and B respectively of medium LCM-1, 100% equals 72 ± 1 and 37 ± 2 colonies (mean $\pm$ S.E.M.). For group A and B respectively of medium LCM-2, 100% equals 72 ± 3 and 19 ± 1 colonies (mean $\pm$ S.E.M.). Four determinations were made for each assay of activity.

Enzyme treatment	Enzyme concn. (μ g/ml)	Enzyme inhibitor	Group	Recovery of colony-stimulating activity (%)	
				Medium LCM-1	Medium LCM-2
Control	None	Phenylmethanesulphonyl fluoride	A	100	100
			B	100	100
Pronase	500	Bovine serum albumin	A	0	0
			B	0	0
Trypsin	1000	Phenylmethanesulphonyl fluoride	A	0	19
			B	0	0
α -Chymotrypsin	500	Phenylmethanesulphonyl fluoride	A	1	19
			B	0	11
Neuraminidase	35	None	A	89	97
			B	96	100

not possible to obtain enough material to carry out such a purification procedure to provide reliable estimates of yield and degree of purification.

Sensitivity of colony-stimulating activity

Evidence has been obtained that colony-stimulating activity from other sources and detected by using cultures of mouse marrow cells may be glycoproteins (Stanley & Metcalf, 1972; Austin *et al.*, 1971); a characteristic feature of these colony-stimulating activities was their relative insensitivity to proteolytic enzymes. To test the sensitivity to enzymes of the activity derived from normal human peripheral leucocytes, two separate preparations of leucocyte-conditioned medium (LCM-1 and LCM-2) were treated with Pronase, trypsin, α -chymotrypsin or neuraminidase. As shown in Table 4, activity in medium LCM-1 was totally inactivated by Pronase and trypsin digestion and markedly inactivated by α -chymotrypsin digestion. With preparations of medium LCM-2, Pronase digestion totally inactivated colony-stimulating activity, whereas trypsin and α -chymotrypsin partially inactivated the preparations to an extent dependent on their stage of purification. None of the preparations showed any significant change in activity after digestion with neuraminidase.

Concluding remarks

The most important finding in the present study is that normal peripheral blood leucocytes liberate three molecular-weight species with colony-stimu-

lating activity into culture media, whereas leucocytes from patients with untreated leukaemia apparently release only a single species. Price *et al.* (1975) observed that colony-stimulating activity may be obtained directly from cell membranes. When this technique was applied to cells from patients with leukaemia, three molecular species of activity were identified in the membranes even when only a single molecular species was detected in culture media (Price *et al.*, 1974). The basis for the difference between observations derived from conditioned media and those derived from cell membranes is not known; studies are in progress to determine whether bioactive molecules, present in the membrane, are degraded or altered as rapidly as they are released into culture media or alternatively if these molecules associated with leukaemic cells are less available for release into culture media.

We thank Ms. K. Benzing, Mr. D. Course, Ms. E. Frojd and Ms. J. Grover for excellent technical assistance. This work was supported by grants from the National Cancer Institute of Canada, the Medical Research Council, Canada (Grant no. MT-1420) and the Ontario Cancer Treatment and Research Foundation (Grant no. 236).

References

- Austin, P. E. (1971) Ph.D. Thesis, University of Toronto
- Austin, P. E., McCulloch, E. A. & Till, J. E. (1971) *J. Cell. Physiol.* **77**, 121-134
- Brown, C. H., III & Carbone, P. P. (1971) *J. Nat. Cancer Inst.* **46**, 989-1000
- Chan, S. H. & Metcalf, D. (1970) *Nature (London)* **227**, 845-847

- Chan, S. H., Metcalf, D. & Stanley, E. R. (1971) *Brit. J. Haematol.* **20**, 329–341
- Chervenick, P. A. & Boggs, D. R. (1970) *Science* **169**, 691–692
- Dacie, J. V. & Lewis, S. M. (1968) *Practical Haematology*, J. and A. Churchill, London
- Iscoe, N. N., Senn, J. S., Till, J. E. & McCulloch, E. A. (1971) *Blood* **37**, 1–5
- Lind, D. E., Bradley, M. L., Gunz, F. W. & Vincent, P. C. (1974) *J. Cell. Physiol.* **83**, 35–42
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–272
- Martin, R. G. & Ames, B. N. (1961) *J. Biol. Chem.* **236**, 1372–1379
- Messner, H. A., Till, J. E. & McCulloch, E. A. (1973) *Blood* **42**, 701–710
- Paran, M., Sachs, L., Barak, Y. & Resnitzky, P. (1970) *Proc. Nat. Acad. Sci. U.S.* **67**, 1542–1549
- Pike, B. L. & Robinson, W. A. (1970) *J. Cell. Physiol.* **76**, 77–84
- Price, G. B., McCulloch, E. A. & Till, J. E. (1973) *Blood* **42**, 341–348
- Price, G. B., McCulloch, E. A. & Till, J. E. (1974) in *The Cell Surface: Immunological and Chemical Approaches* (Kahan, B. & Reisfeld, R., eds.), pp. 237–241, Plenum Press, New York
- Price, G. B., McCulloch, E. A. & Till, J. E. (1975) *Exp. Haematol.* in the press
- Sheridan, J. W. & Metcalf, D. (1973) *J. Cell. Physiol.* **81**, 11–24
- Sheridan, J. W. & Stanley, E. R. (1971) *J. Cell. Physiol.* **78**, 451–460
- Stanley, E. R. & Metcalf, D. (1971) *Proc. Soc. Exp. Biol. Med.* **137**, 1029–1031
- Stanley, E. R. & Metcalf, D. (1972) in *Cell Differentiation* (Harris, R. & Viza, D., eds.), pp. 272–276, Munksgaard, Copenhagen