COLONY-STIMULATING FACTOR FROM UMBILICAL CORD

ENDOTHELIAL CELLS

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# COLONY-STIMULATING FACTOR FROM UMBILICAL CORD ENDOTHELIAL CELLS

THESIS

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Conditioned media prepared from umbilical cord (UC) segments or endothelial cells (EC) contain colony stimulating activity. Both UCCM and ECCM were partially purified by DEAE-Sepharose and ACA44 gel filtration chromatography. The molecular weights were estimated as 25,000 and 31,000 for UC-CSF and EC-CSF, respectively. UC-CSF was further fractionated by Con A Sepharose, IEF and HPLC on a hydrophobic phenyl column. The highly purified CSF stimulates human macrophage and granulocyte colony formation, indicating it is GM-CSF in nature. Characterization studies have revealed that both CSFs are heat stable at 60°C for 30 min. They are sensitive to digestion by protease and to periodate oxidation but are stable to treatment with sulfhydryl reagents. The synthesis of CSF in endothelial cells is inhibited by actinomycin D, cycloheximide and puromycin, indicating that protein and RNA synthesis are required for CSF production. Among the mitogens tested, only LPS exhibited stimulatory activity on the production of CSF. Metabolic modulators such as dibutyryl cAMP, isobutylmethylxanthine, $PGE_2$ and lactoferrin inhibit CSF production, while $PGF_2$ enhances CSF production.

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## ABBREVIATIONS

| | |
|---|---|
| UCCM | Umbilical cord-conditioned medium |
| ECCM | Endothelial cell-conditioned medium |
| UC-CSF | Umbilical cord - colony-stimulating factor |
| EC-CSF | Endothelial cell - colony-stimulating factor |
| CSF | Colony-stimulating factor |
| IL-3 | Interleukin-3 |
| PSF | P-cell-stimulating factor |
| BPA | Burst-promoting activity |
| HCGF | Hemopoietic cell growth factor |
| GM-CSF | Granulocyte-macrophage colony-stimulating factor |
| MGI-1GM | Macrophage-granulocyte inducer-factor 1 granulocyte-macrophage |
| G-CSF | Neutrophilic granulocyte-colony-stimulating factor |
| MGI-1M | Macrophage-granulocyte inducer-factor 1 granulocyte |
| MIAPaCa-2 | Miami pancreatic carcinoma cell line |
| BFU-E | Burst-forming unit-erythroid precursor |
| CFU-mix | Colony forming unit-progenitor cell |
| cAMP | Cyclic adenosine 3':5'-monophosphate |
| cGMP | Cyclic guanidine 3':5'-monophosphate |
| CIA | Colony inhibitory activity |
| PG | Prostaglandin |
| Con A | Concanavalin A |

| | |
|---|---|
| Mr | Apparent molecular weight |
| DME | Dulbecco's modification of Eagles medium |
| PBS | Phosphate-buffered saline, pH 7.4 |

# INTRODUCTION

Colony-stimulating factor (CSF) is a hormonal protein factor required for the *in vitro* growth of granulocyte and macrophage colonies (1) from bone marrow stem cells and possibly functions as a granulopoietin *in vivo* (2,3). Recent progress in our understanding of hematopoiesis clearly indicates that the peripheral blood cells, including erythrocytes, granulocytes, macrophages, mast cells and lymphocytes, are all derived from a common stem cell and the control of this differentiation process is regulated by different hormonal growth factors. Thus multi-CSF, also known as interleukin-3 (IL-3) (4), stimulates the proliferation of erythrocytes, neutrophilic granulocytes, megakaryocytes, eosinophilic granulocytes and mast cells. Because of its diversified functions, many other names have been used in different assays, including P-cell stimulating factor (PSF) (5), burst-promoting activity (BPA) (6), hemopoietic cell growth factor (HCGF) (7), stem cell activating factor (8) and mast cell growth factor (9). Granulocyte-Macrophage colony-stimulating factor (GM-CSF), also known as macrophage-granulocyte inducer factor 1 granulocyte-macrophage (MGI-1GM) (10), is specific for the formation of macrophage and neutrophilic granulocyte colonies. Neutrophilic granulocyte CSF (G-CSF), also known

as macrophage-granulocyte inducer factor 1 granulocyte (MGI-1G) (10), stimulates the formation of neutrophilic granulocyte colonies. Macrophage colony-stimulating factor (M-CSF) (11), also known as CSF-1 or macrophage-granulocyte inducer factor 1 (MGI-1M) (10), stimulates macrophage colony formation. In addition, all of the CSFs studied to date exhibit a capacity to stimulate the proliferation of cells in other lineages, but this capacity varies widely in magnitude. For example, multi-CSF is an effective proliferative stimulus for a number of different blood cells except lymphocytes, while CSF-1 exhibits no additional action other than an ability in conjunction with hemopoietin I to stimulate undefined progenitor cells with high proliferative potential toward the macrophage lineage (12). At high concentrations, GM-CSF is an effective proliferative stimulus for eosinophils, megakaryocytes and mixed erythroid cells (13). At relatively low concentrations, GM-CSF can initiate, but not sustain, cell division in multipotential, erythroid, and megakaryocytic progenitors. On the other hand, G-CSF, at all concentrations tested so far, has no proliferative action on eosinophil or stem cells but can initiate proliferation in multipotential and erythroid progenitors. The latter is a weaker action than that exhibited by GM-CSF (14).

Colony-stimulating factor can be isolated from tissue fluid, conditioned media from a variety of tissues and

cultured cancer cells. In our laboratory human CSFs have been isolated and purified from the conditioned media of human pancreatic carcinoma cell line (MIAPaCa-2) (15), human lung (16) and human placenta (17). With advances in the techniques of high performance liquid chromatography (HPLC) and the gas phase micro sequencer, several CSFs including murine GM-CSF (18,19), murine CSF-1 (20,21), murine G-CSF (22), murine multi-CSF (23-25), human GM-CSF (26), human CSF-1 (27) and human G-CSF (27,28) have been purified and partial amino acid sequences determined. Based on the sequence of the N-terminal region obtained from purified CSFs, oligonucleotide probes have been synthesized and used to isolate the cDNA for these CSFs (27,34,35,36). The total amino acid sequence of mouse GM-CSF (30), mouse multi-CSF (31,32), human GM-CSF (26,33,34), human CSF-1 (27) and human G-CSF (29,35,36) have been derived from their respective cDNA. Furthermore, the recombinant murine GM-CSF, human GM-CSF and human G-CSF have been produced in large quantity and have exhibited activity similar to that of the native CSFs. The biological and biochemical characterization of these CSFs have been reported recently (37-42).

Although many tissues and cell lines synthesize and secrete a CSF, work on the regulation of biosynthesis of CSF has been very limited. Endotoxins and lectins stimulate CSF production in a number of CSF-producing cells (44,45), whereas lactoferrin inhibits production (56-58). The

mechanisms involved in the regulation of CSF synthesis have yet to be investigated.

Several questions can be raised in this regard. For example, is the heterogeneity of CSF due to multiple-gene products or a function of post-translational modifications? If there are multiple genes, how many are there? How are the genes organized and regulated? If heterogeneity is a result of post-translational events, is there a pre-CSF product? How are the carbohydrate moieties attached to the peptide? How are CSFs synthesized and secreted? The structural studies of CSF-1 have revealed that it is an N-glycoprotein and that a post-translational processing occurs at the C-terminus (27). In addition, a recent report by Souza et al has shown that G-CSF is an O-glycoprotein with a short oligosaccharide side chain (36). Although the MIAPaCa-2 cell is an excellent producer of CSFs, it does not respond to regulators such as endotoxin and lactoferrin. It is therefore necessary to establish a CSF-production system other than the MIAPaCa-2 cell to investigate the regulation of CSF biosynthesis. Segments of human umbilical cord have been used to prepare a highly active conditioned medium (UCCM) by Irvine et al (46). Colonies derived from UCCM stimulation consisted of over 98% granulocytic cells by morphological and cytochemical analysis. Endothelial cells (EC) are components of human marrow stroma and have been identified as constituents of the adherent layer in

long-term cultures of human and mouse marrow. Although the *in vivo* role of the EC in hematopoiesis is not known, the secretion of human CSF by UC-EC has been reported (47,64). Ascensao *et al* have reported that endothelial cells produce proteins of Mr 30,000 and isoelectric points of 4.5 and 7.2 that stimulate the growth of human Burst-Forming Unit-Erythroid (BFU-E) and Colony-Forming Unit-Mix (CFU-mix). A heat-labile protein has also been isolated that stimulates the proliferation and differentiation of granulocyte-macrophage progenitor cells (CFU-GM). No erythropoietin was detected in endothelial cell supernatants (47). These data suggest that endothelial cells may play an active role in the modulation of hematopoiesis. In spite of these studies, UC-CSF and EC-CSF are still not well characterized.

Although CSF is required for colony growth, a variety of substances, particularly low-molecular weight compounds, are capable of enhancing or inhibiting CSF-induced colony formation. Cyclic adenosine-3':5'-monophosphate (cAMP) and prostaglandin $E_2$ (48,49) inhibit colony growth in both mouse and human marrow, assay whereas cGMP enhances colony formation (50). The effect of prostaglandin (PG) on CFU-GM growth has also been extensively studied (51-55). Kurland *et al* have described that the production of $PGE_2$ by macrophages causes the inhibition of CSF production. Suppression of $PGE_2$ synthesis by indomethacin markedly

increases production of CSF, thus enhancing colony formation (55). The role of CSF and $PGE_2$ as a positive and negative feedback control of myeloid differentiation has also been proposed. Miller et al examined the role of the F series of prostaglandin and found that $PGF_2$ enhances colony growth (56). However, the effect of $PGF_2$ on CSF production has not been reported. Extensive studies on the inhibitory effect of lactoferrin on myelopoiesis have been conducted and reported by Broxymeyer and his coworkers (57-59). Lactoferrin is an iron-binding glycoprotein concentrated in the secondary granules of mature granulocytes. It was originally named colony-inhibitory activity or CIA (57) but later identified as lactoferrin (58). Lactoferrin exerts its inhibitory action on colony formation by inhibiting the production and/or release of CSF from monocytes (59).

In this study, UC-CSF and EC-CSF were isolated and partially purified from UCCM and ECCM. They were then characterized and compared. Endothelial cells in culture were used as a CSF-producing cell system to study the regulation of CSF synthesis. Macromolecular inhibitors, mitogens, cyclic nucleotides, prostaglandins and iron proteins were used to study their effect on CSF biosynthesis and to elucidate the possible mechanisms involved in the regulation of CSF production.

## MATERIALS AND METHODS

Assay of CSF biological activity: The soft agar assay of mouse bone marrow cells was carried out as described previously (16). Bone marrow cells were obtained from the femora of C57BL/6J (Charles River Breeding Lab., Stone Ridge, MA) inbred mice. Bone marrow cells were expelled by inserting a 21 1/2 gauge needle into the proximal end and forcibly rinsing out the content with 3 ml of ice-cold DME medium. The cell suspension was evenly dispersed by gently aspirating up and down 10-15 times using the syringe. An aliquot of cell suspension was diluted 1 to 20 in 1.5 ml of glacial acetic acid and shaken in a pipettor shaker for 1 min. The number of nucleated cell was counted using a hemocytometer. The mononuclear cells (density<1.007 g/ml) from human cord blood were isolated by density centrifugation on Ficoll Paque (Pharmacia) and were depleted of adherent cells by two- to three-hour incubations in tissue culture dishes. The nonadherent mononuclear cells were cultured at a concentration of $2x10^5$ cells per ml of the assay medium. For the culture medium, double strength DME medium containing 20% fetal calf serum, 20% horse serum, 19.8 ug/ml of asparagine, 75 ug/ml of DEAE Dextran, 100 units/ml of penicillin, 100 ug/ml of streptomycin and 7.6 mg/ml of sodium bicarbonate was mixed

with an equal volume of 0.6% agar at 37$^0$C. The bone marrow cells were immediately added to this culture medium at a final cell concentration of 100,000 nucleated cells/ml. One ml of cell suspension was dispensed into each 35 mm petri dish containing 0.2 ml of sample to be assayed. The dishes were then swirled to ensure an even distribution and left at room temperature for 30 min to allow the agar to gel. Dishes were then incubated at 37$^0$C under 6% $CO_2$ for 5 days. Aggregates of 50 or more cells were counted as colonies using a dissecting microscope. One unit of CSF activity is defined as the amount of activity that stimulates the formation of one colony under the specified conditions of the assay.

Morphological Analysis: The study of cell morphology in assay plates was carried out by a technique developed in this laboratory (60). Briefly, the soft agar plates containing colonies are fixed with 30% acetic acid in absolute ethanol for 20 min at room temperature. Each plate is then incubated sequentially with 100, 100, 80, 80, 50, and 50% ethanol for 10 min each. The fixed agar layer is removed by floating on water and dried at room temperature overnight. The dried thin film is stained with hematoxylin for 10 min, then rinsed with running tap water for 3 min to remove excess dye. The film is air-dried and examined under the microscope for cell morphology of the individual

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colonies.

Preparation of Umbilical Cord Conditioned Medium:

Samples of human umbilical cord were collected in a sterile container, filled with M199 serum-free medium, and maintained at $4^0$C until processing. Pieces approximately 0.5 cm long were cut from the cord and rinsed with PBS solution. Segments were suspended in 500 ml M199 serum-free medium containing 5 ug/ml of Amphotericin B, 5 ug/ml of lipopolysaccharide, 5 ug/ml of phytohemagglutinin, 5 ug/ml of Con A, and 0.1 mM phenylmethylsulphonylfluoride in roller culture bottle. Bottles were incubated at $37^0$C for seven days, as this was found to be the optimal time for the production of stimulating activity. The conditioned medium was harvested as the supernatant after centrifugation at 10,000 rpm for 30 min (Beckman, JA-20 rotor) to remove debris, and the supernantant was concentrated using a Hollow Fiber concentrator with HIP5-20 cartridge, dialyzed against $H_2O$ and then stored at $-20^0$C for future use.

Isolation of Endothelial Cells: Endothelial cells were obtained from human umbilical cord veins by an adaptation of the method of Gimbrone and Cotran (59). All procedures were carried out under sterile conditions. The cord was severed from the placenta soon after birth, placed in a sterile container filled with M199 serum-free medium, and kept at $4^0$C until processing. The cord was inspected, and all

areas with clamp marks were excised. The umbilical vein was cannulated with an 18 1/2 gauge needle, and the tip of needle was protected with PE 160 tubing. The vein was then perfused with 20 ml of M199 media to wash out the blood and allowed to drained. One end of the cord was clamped with a hemastat and 10ml of 0.1% collagenase (4196, Cooper Biomedical) in M199 serum-free media was infused into the umbilical vein from the other end, which was then also clamped with a hemastat. The umbilical cords were incubated for 18 min under the hood at room temperature and kneaded back and forth to expose the entire cord to collagenase solution. After incubation, the collagenase solution containing the endothelial cells was flushed from the cord by perfusion with 20 ml of M199 serum-free medium. The effluent was collected in a sterile 50 ml conical centrifuge tube (25330, Corning). The cells were centrifuged at 250 x g for 10 min and washed twice with 10ml of M199 supplemented with 20% human serum. The cell pellet was then resuspended in 2 ml of fresh 20% human serum containing M199 medium.

Cultured Endothelial Cells: The cell suspension prepared in the previous section was cultured in 60 x 15 mm dishes (Falcon 3802 , Primaria culture dish) with M199 medium containing 20% human serum, 90 ug/ml heparin, 25 ug/ml Endothelial Cell Growth Supplement (40006, Collaborative Research, INC) and 5 ug/ml Amphotericin B. The

dishes were incubated at $37^0$C under 6% $CO_2$.

The monolayer of endothelial cells were cultured under these conditions with the growth medium changed every 72 hours. The CSF-containing growth medium was collected and pooled for further fractionation.

Subculture of Endothelial Cells: The confluent cultured endothelial cells were scrapped with a rubber policeman and split 1:3 in culture dishes (60x15 mm). Culture conditions were the same as above.

Chromatography of CSF on a DEAE Sepharose CL-6B column: Serum-free conditioned medium from umbilical cord (UCCM) was concentrated by ultrafiltration with an Amicon stirred cell concentrator (PM-10 membrane) and dialyzed against distilled water overnight. The dialyzed sample was applied to a DEAE Sepharose CL-6B (Pharmacia) column (2.1 x 16 cm), equilibrated in 0.01 M Tris-HCl, pH 7.6, containing 0.01% $NaN_3$, 0.05 M NaCl, and 0.01% polyethylene glycol (PEG, Mr 3,350). After loading the sample, the DEAE column was eluted with a 500 ml linear gradient 0.05 M to 0.2 M NaCl in 0.01 M Tris-HCl, pH 7.6, followed by 100 ml of 0.3 M NaCl in the same buffer. The flow rate was 50 ml/hr. Aliquots of 2 ml from every other fraction were desalted using a PD-10 column (Pharmacia), sterilized by filtration and assayed for CSF activity.

Chromatography of CSF on a ACA44 gel filtration column: The active fractions from the DEAE-Sepharose column were pooled and concentrated by ultrafiltration with an Amicon stirred cell concentrator (PM-10 membrane), and loaded into the ACA44 gel filtration column (3 x 118 cm, Ultragel LKB 2204-440). The column was eluted with 0.01 M Tris-HCl, pH 7.6, with 0.3 M NaCl, 0.01% $NaN_3$, and 0.01% PEG. The flow rate was 27 ml/hr, and 5 ml fractions were collected. Aliquots of 2 ml from every other fraction was assayed for CSF activity.

Chromatography of CSF on a Concanavalin A-Sepharose 4B column: The active fractions from the ACA44 column were pooled, concentrated by ultrafiltration as above, dialyzed overnight against buffer containing 0.1 M sodium acetate, pH 6.0 with 0.5 M NaCl, 10 mM $MnCl_2$, 10 mM $MgCl_2$, 10 mM $CaCl_2$, 0.04% $NaN_3$, and 0.04% PEG. This dialyzed sample was then applied to a Con A-Sepharose 4B column (2 x 6 cm) equilibrated with the same buffer. After loading the sample, the column was washed with 50 ml of buffer following by 50 ml of the same buffer with 0.5 M $\alpha$-methyl-D-mannoside . The flow rate was 20 ml/hr and 1.6 ml fractions were collected. Aliquots of 0.05 ml from every other fraction were desalted and assayed for CSF.

Fractionation of CSF from the Con A Sepharose Pool by Preparative Isoelectrofocusing: The $\alpha$ -methyl-D-mannoside

eluted fractions were pooled, concentrated by ultrafiltration as above and dialyzed against distilled water. This sample was then subjected to isoelectrofocusing on an LKB 2117 multiphor preparative electrofocusing apparatus according to a standard procedure (LKB manual # 1-2117-501-E01). The pH gradient was from 3.5 to 5 and a gel bed of 100 ml Sephadex IEF (Pharmacia) was used. The voltage, current and power were set at 1,200 volt, 16 mA and 8.0 watts, respectively. After the isoelectrofocusing, the gel bed was divided into thirty fractions and the gel in each fraction was poured into a column. The column was eluted first with 2 ml of distilled water for pH determination followed by 6 ml of PBS buffer, pH 7.4. Aliquots of every fraction were then assayed for CSF activity.

Chromatography of CSF on HPLC TSK Phenyl-5PW column:

The active fractions from PIEF were pooled, concentrated and dialyzed against the TSK phenyl column initial buffer (0.01 M sodium phosphate, pH 6.9, containing 1.1 M ammonium sulfate, 0.02% $NaN_3$ and 0.02% PEG). The sample was then applied to a HPLC TSK Phenyl-5-PW column (7.5 x 75 mm, BIO-RAD, Richmond, CA). The HPLC system consists of two 1330 pumps, gradient mixer, gradient processor system software, ISCO UA-5 monitor and Rheodyne 7125 injector. After injecting the sample, the column was eluted with eluting

buffer of 0.01 M sodium phosphate, pH 6.8, containing 0.02% $NaN_3$, 0.02% PEG and 3% isopropanol, pH 6.8. The flow rate was maintained at 1 ml/min and 2 ml fractions were collected. The CSF activity of each fraction was assayed.

Temperature Stability: Aliquots of CSF (500 units) in 1 ml PBS containing a $50^0$C, $60^0$C, $80^0$C for 30 min and $100^0$C for 5 min. Samples were chilled in ice after incubation, desalted by PD-10 column, sterilized by filtration and assayed.

Protease Digestion: Stock solutions of trypsin, chymotrypsin, and subtilisin were prepared by dissolving 10 mg each of the enzyme in 1 ml of 0.01 M Tris-HCl, pH 8.0, with 0.01 M calcium chloride. Aliquots of CSF (500 units), in 4 separate 1 ml volumes of the Tris-calcium chloride buffer, were prepared and 50 ug of one of the enzymes from the stock solution or buffer was added and solutions were incubated at $37^0$C for 3 hours. At the end of the incubation, the sample was chilled on ice immediately and 0.1 ml of fetal calf serum was added to the incubation mixture. The sample was assayed for CSF activity.

Periodate Treatment: Sodium periodate at the concentration of 1, 5 and 10 mM in 0.1 M sodium phosphate, pH 7.0 buffer, was incubated with aliquots CSF (500 units) in a final volume of 1 ml at $37^0$C for 3 hours. At the

end of the incubation, the sample was processed as above and assayed for CSF activity.

Sulfhydryl Reagent Treatment: Dithiothreitol at concentration of 1, 5 and 10 mM was incubated with aliquots of CSF (500 units) in 1 ml of 0.1 M sodium phosphate, pH 7.0, at $37^0$C for 3 hours. At the end of the incubation, the samples were treated as above and assayed for CSF activity.

Effect of Macromolecular Inhibitors on CSF Production: Cytosine arabinoside, actinomycin D, cycloheximide and puromycin at concentrations of 0.5, 1, 5, 10 and 100 ug/ml were added to the endothelial cells in serum-free M199 medium. After seven days, the media were harvested, deaslted on a PD-10 column, sterilized by filtration and assayed for CSF activity at 200 ul/plate in triplicate. For this and following experiments, endothelial cells were cultured until confluent. The growth medium was removed by aspiration and the plates were washed twice with PBS and 1 ml of serum-free medium was then added to each plate for the specific experiment.

Effect of Mitogen on CSF Production: Different mitogens were added to the endothelial cells in serum-free M199 medium at a concentration of 5 ug/ml. Incubation was continued for 7 days and the media were harvested and

assayed for CSF activity.

Effect of Cyclic Nucleotides on CSF Production: Endothelial cells in serum-free M199 medium were incubated with dibutyryl cAMP, dibutyryl cGMP and isobutylmethylxanthine at the concentration of 0, $10^{-7}$, $10^{-8}$ and $10^{-9}$ M for 7 days. The incubation mixtures were harvested and assayed for the CSF activity.

Effect of Arachidonic Acid Metabolites on CSF Production: Cultured endothelial cells in serum-free M199 medium were incubated with 0, $10^{-6}$, $10^{-8}$, $10^{-9}$ M of prostaglandin $E_2$ or $F_2$, in final volume of 1 ml. The stock solution (100X) of the prostaglandin $E_2$ and $F_2$ were prepared in absolute ethanol. After another seven days culture, the media were harvested and assayed for CSF.

Effect of Iron Proteins on CSF Production: To petri dishes with confluent cultured endothelial cells was added serum-free M199 medium containing 0, $10^{-6}$, $10^{-7}$, $10^{-8}$, and $10^{-9}$ M iron-saturated lactoferrin or transferrin, in a final volume of 1 ml. Iron-saturated lactoferrin and transferrin were prepared by dialyzing the proteins for 48 hours at $4^0$C, first against 500 volumes of saturated ferrous ammonium sulphate ($Fe(NH_4)2(SO_4)_2.6H_2O$) and then against PBS at $4^0$C as described previously (58). The incubations

were maintained for seven days and media were harvested and assayed for CSF.

## RESULTS

### Partial Purification of CSF from UCCM

Human Umbilical Cord segments when incubated in M199 serum-free medium produced CSF activity continuously up to seven days as shown in Fig.1. Under the specified conditions described in the Methods, the CSF activity at day 7 is about 400 units/ml although the activity produced varied from one preparation to the other. A summary of the purification of CSF from UCCM is shown in Table I. The UCCM was concentrated using a Hollow-Fiber concentrator and Amicon ultrafiltration and dialyzed exhaustively against water before application to a DEAE-Sepharose column as described in the Methods. As shown in Fig.2 , the majority of the activity was eluted between 0.10 and 0.13 M NaCl. A 1.4-fold purification and 63% recovery was achieved. No activity was detected either in the break through fractions of 0.05 M NaCl or in the high salt wash (0.5 M NaCl) fractions. The active fractions from Fig.2 were concentrated and loaded onto a ACA 44 column as shown in Fig.3. Most of the activity appeared as a broad peak around fraction 80 which has an Mr of 25,000. Since the activity was eluted with a low amount of protein, this provided a good purification step. It had 4-fold purification and 8.2% recovery. As shown in Fig.4,

Fig. 1 - Time course of CSF production in UCCM. Human umbilical cord was sliced into small segments and incubated in 500 ml M199 serum-free medium at 37°C. Aliquots (200 ul) of samples were taken at designate time intervals and assayed for CSF activity with mouse bone marrow cells.

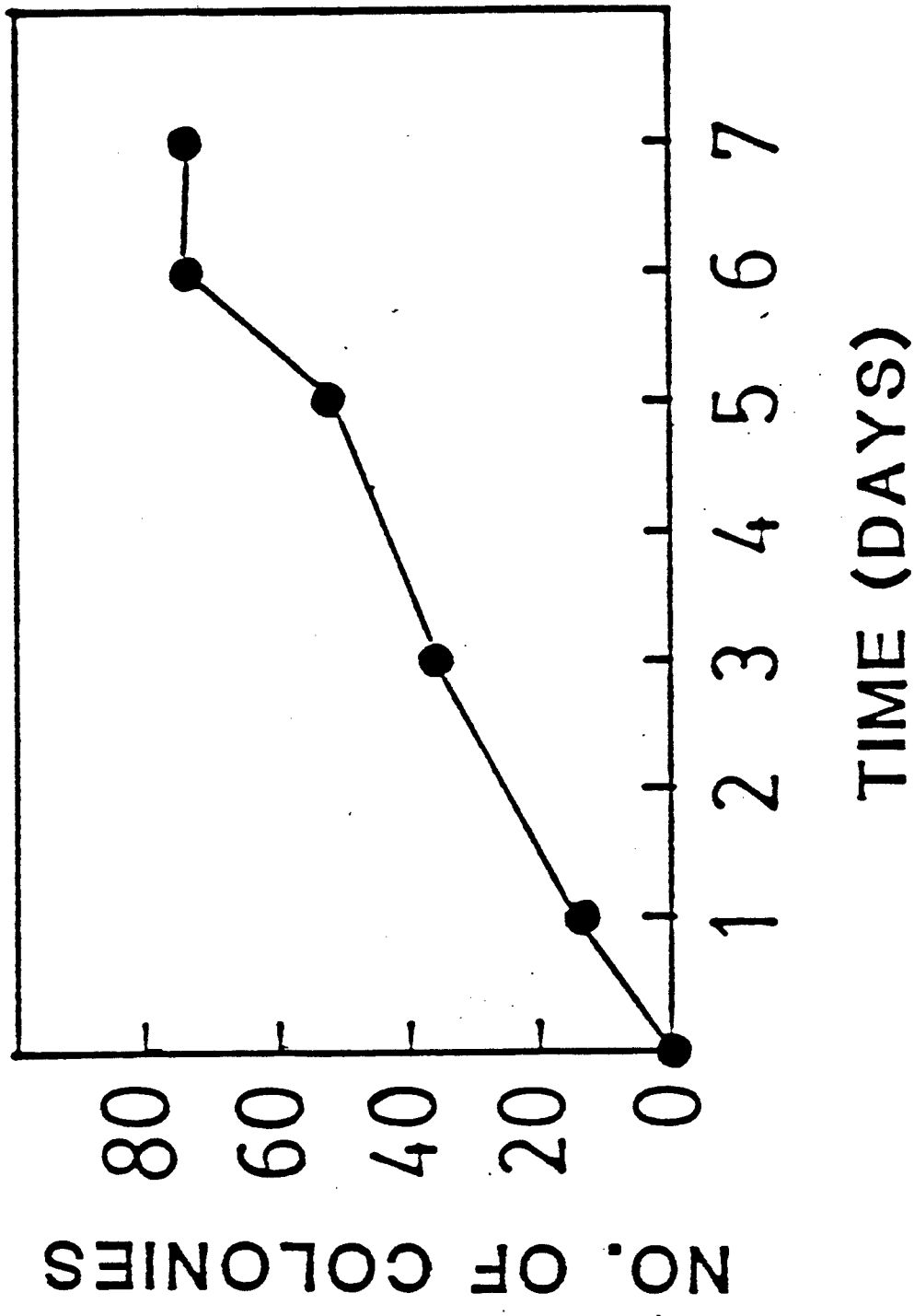

NO. OF COLONIES
0
20
40
60
80
TIME (DAYS)
1
2
3
4
5
6
7

TABLE I

PURIFICATION OF UMBILICAL CORD-COLONY STIMULATING FACTOR

| STEP | PROTEIN (mg) | TOTAL ACTIVITY (units)* | SPECIFIC ACTIVITY (units/mg) | PURIFICATION (fold) | YIELD (%) |
|---|---|---|---|---|---|
| CONDITIONED MEDIA | 2000 | 507,000 | 253 | 1.00 | 100 |
| DEAE-SEPHAROSE | 874 | 320,000 | 366 | 1.44 | 63 |
| ACA 44 | 44 | 41,580 | 945 | 3.73 | 8 |
| CON A-SEPHAROSE | 11 | 21,120 | 1886 | 7.44 | 4 |
| IEF | 7 | 15,775 | 2320 | 9.15 | 3 |
| HPLC-TSK | 0.2 | 7,000 | 35000 | 138 | 1 |

* One unit is defined as one colony forming in bone marrow assay.

Fig. 2 - Fractionation of UC-CSF by DEAE Sepharose CL-6B column chromatography. The UCCM prepared from seven-day cultures (500 ml) was concentrated, dialyzed and loaded onto a DEAE Sepharose CL-6B column (2.1 x 16 cm). A linear gradient of 500 ml NaCl from 0.05 M to 0.2 M in 0.01 M Tris-HCl, pH 7.6, was used to develop the column. Samples (150 ml) from eluted fractions were desalted by PD-10, sterilized by filtration and assayed for CSF activity as described. Number of colonies is expressed by O---O .

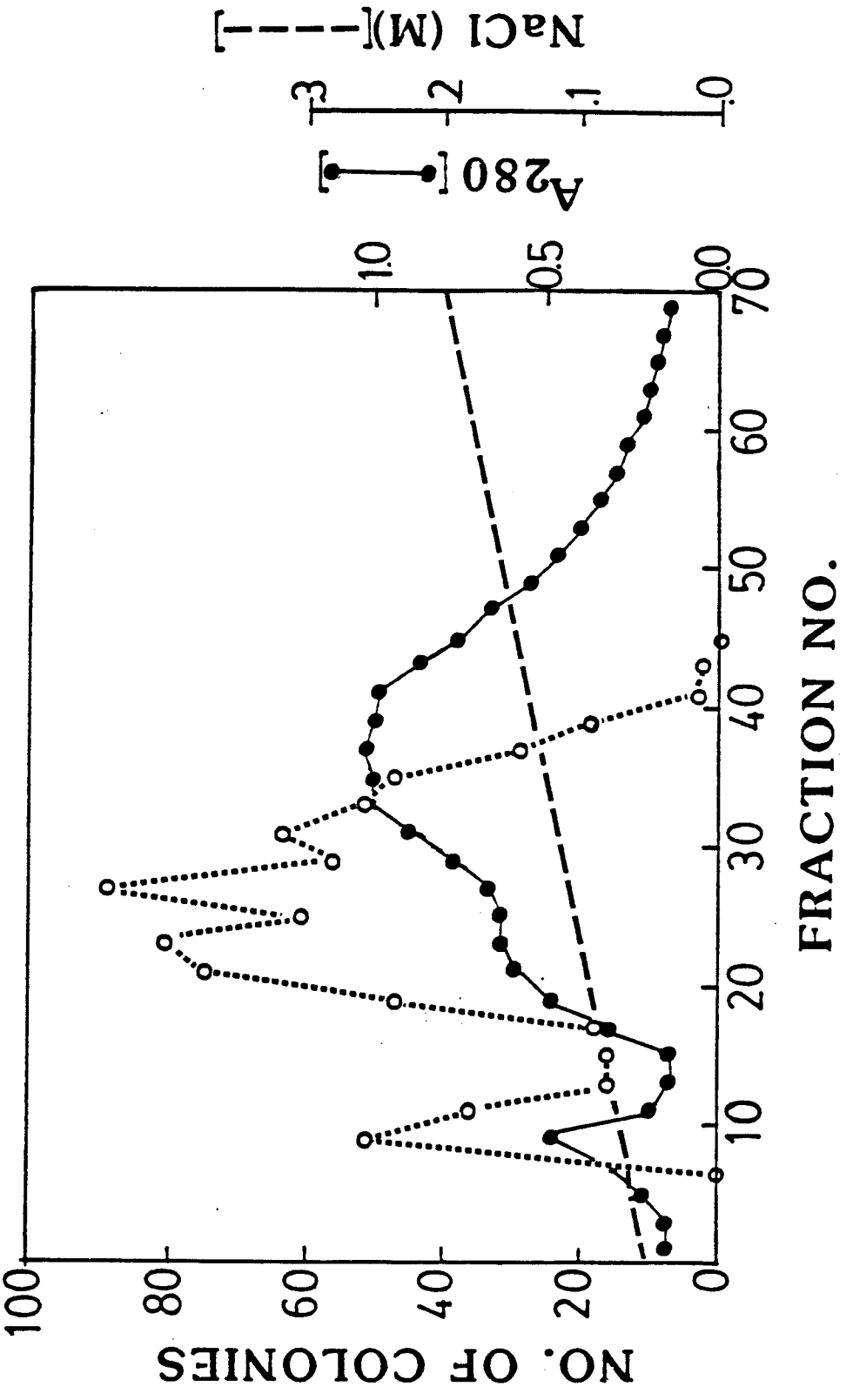

NO. OF COLONIES
100
80
60
40
20
0
FRACTION NO.
0
10
20
30
40
50
60
70
0.0
0.5
1.0
A280 [———]
0
1
2
3
NaCl (M)[----]

Fig. 3 - Fractionation of UC-CSF by ACA44 gel filtration column chromatography. Active fractions from the DEAE column (150 ml) were pooled, concentrated, and loaded onto a ACA44 column (3 x 118 cm). The column was eluted with 0.3 M NaCl in 0.01 M Tris-HCl buffer, pH 7.6. Samples (100 ml) from the eluted fractions were desalted by PD-10, sterilized by filtration, and assayed for CSF activity as described. Number of colonies is expressed by O---O .

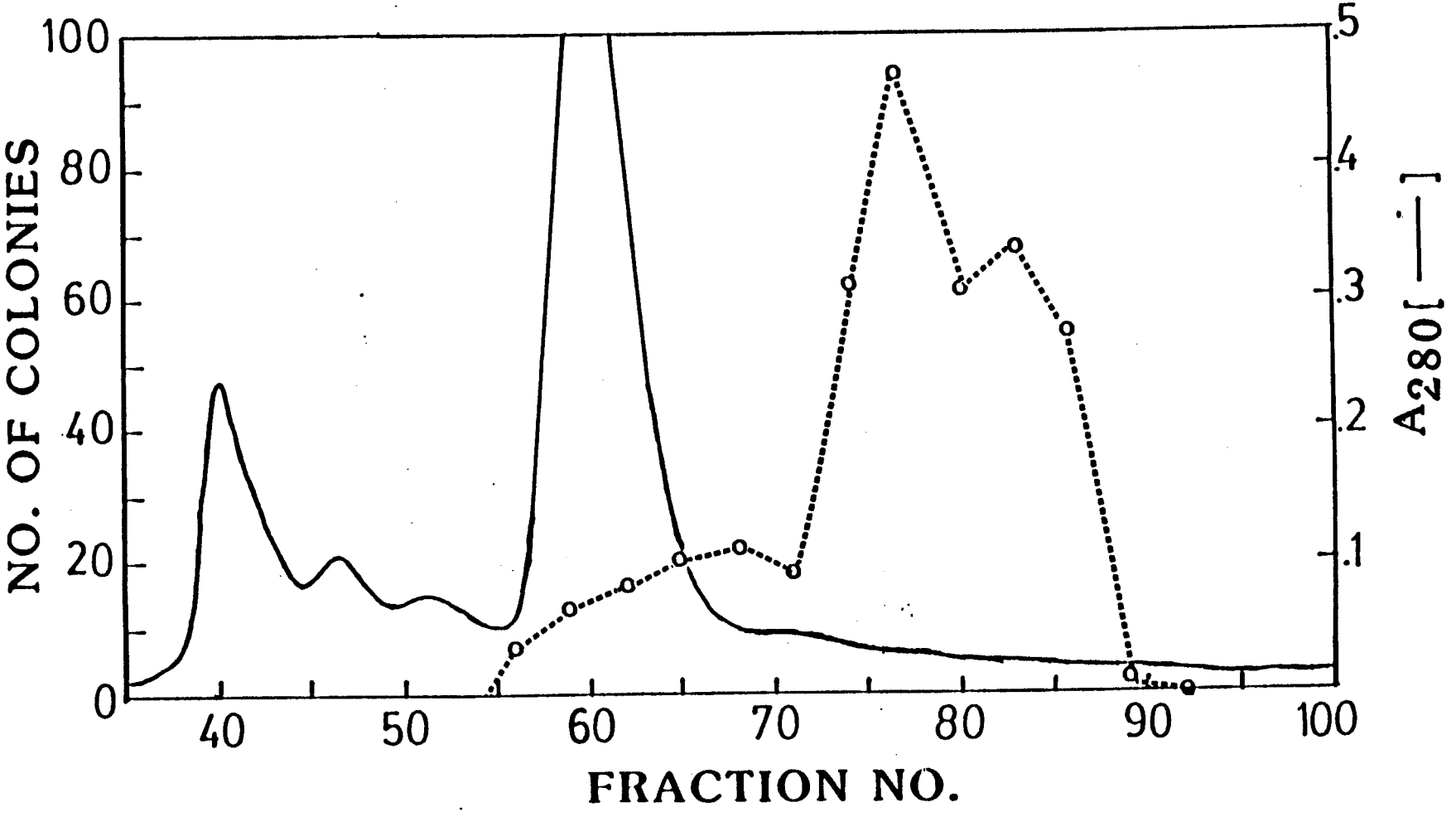
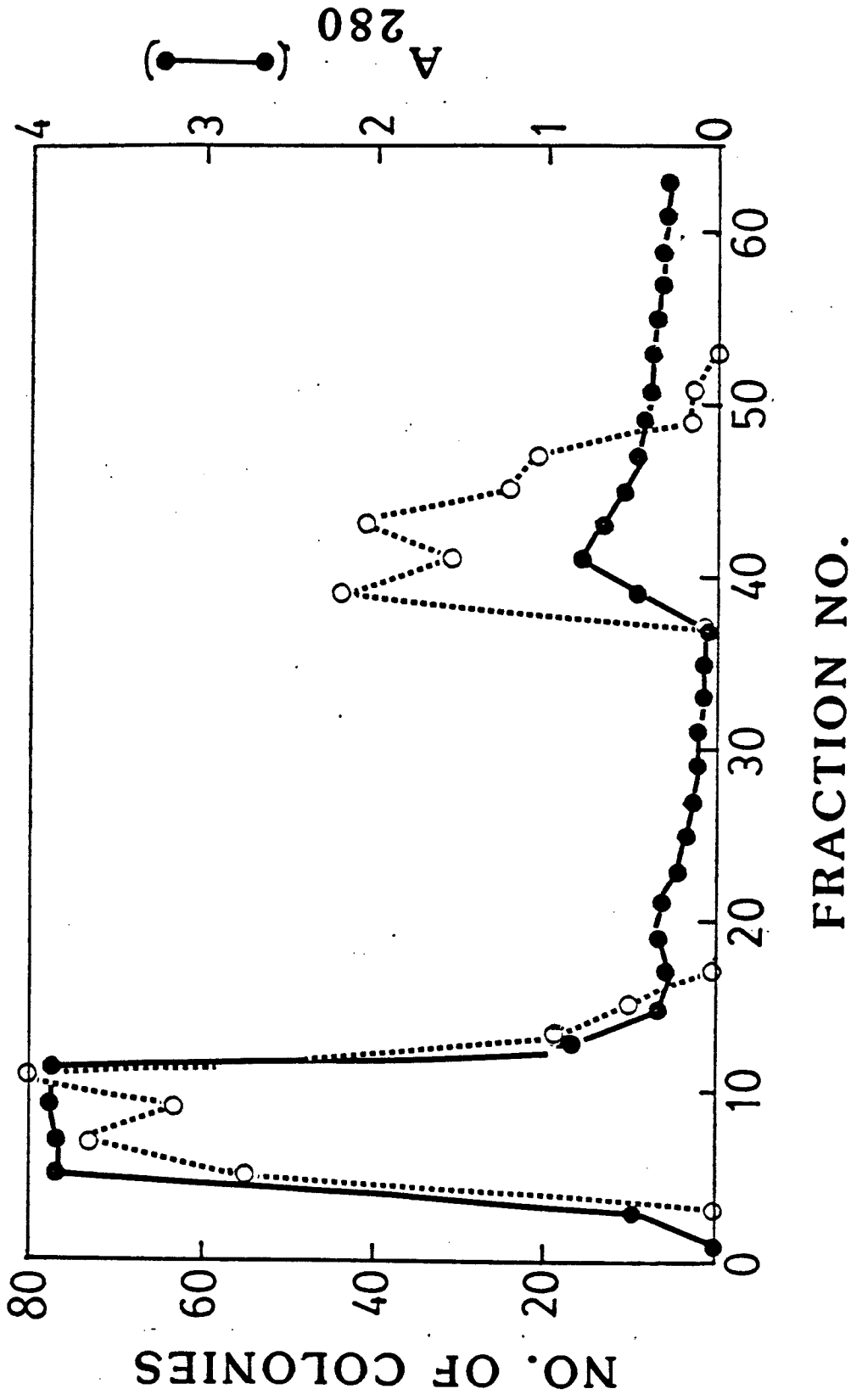

NO. OF COLONIES
100
80
60
40
20
0
40
50
60
70
80
90
100
FRACTION NO.
.5
.4
.3
.2
.1
A280[ —— ]

Fig. 4 - Fractionation of UC-CSF by ConA- Sepharose 4B column chromatography. Fractions containing CSF activity from the ACA44 column chromatography were pooled, concentrated, dialyzed and then loaded onto Con A-Sepharose 4B column (2 x 6 cm). The column was eluted with 0.1 M sodium acetate, pH 6.0, with 0.5 M NaCl, 10 mM $MgCl_2$, 10 mM $CaCl_2$, 0.04% $NaN_3$, and 0.04% PEG (Fraction 1-36), followed by the same buffer with 0.5 M -methyl-D-mannoside (Fraction 37-60). Samples (24 ml) from the eluted fractions were desalted by PD-10, sterilized by filtration and assayed for CSF activity as described. Number of colonies is expressed by O---O .


A 280
NO. OF COLONIES
FRACTION NO.
0
20
40
60
80
0
1
2
3
4
10
20
30
40
50
60

most of the CSF activity appeared in the break through fractions along with bulk of the protein in the Con A-Sepharose profile. Only 30% of the activity was bound to the column and eluted by α-methyl-D-mannoside. The break through fractions were pooled and reloaded onto the column. However, again only a very small amount ( 30%) of CSF activity bound and was eluted by α-methyl-D-mannoside. The purification was 7.4-fold and the recovery was 4.2%. No further attempt to purify the break through fraction was made. The α-methylmannoside-eluted activity from the Con A-Sepharose column was concentrated, dialyzed and further purified by flat-bed isoelectrofocusing on Sephadex gel. The activity appeared as a single peak at pH of 4.5 (Fig.5) indicating an acidic pI for this CSF. The purification at this step was 9.2 fold and recovery was 3.1%. The CSF activity isolated from isoelectrofocusing was further fractionated by HPLC on a TSK phenyl-5-PW column. As indicated in Fig.6, the activity was eluted at about 0.7-0.9 M $(NH_4)_2SO_4$. The CSF activity purified as discussed above was assayed using human bone marrow cells and found active in stimulating macrophage and granulocytic colony formation. At the last step, purification was 138-fold and the yield was 1.4%.

## Partial Purification of CSF from ECCM

Established endothelial cells in culture produce CSF

Fig. 5 - Isoelectrofocusing of UC-CSF. The fractions eluted from Con A-Sepharose 4B with methylmannoside (24 ml) were pooled, concentrated, dialyzed, and further purified by flat-bed isoelectrofocusing on Sephadex gel. Isoelectrofocusing was carried out for 6 hours at 1200 volts. Number of colonies is expressed by O----O .

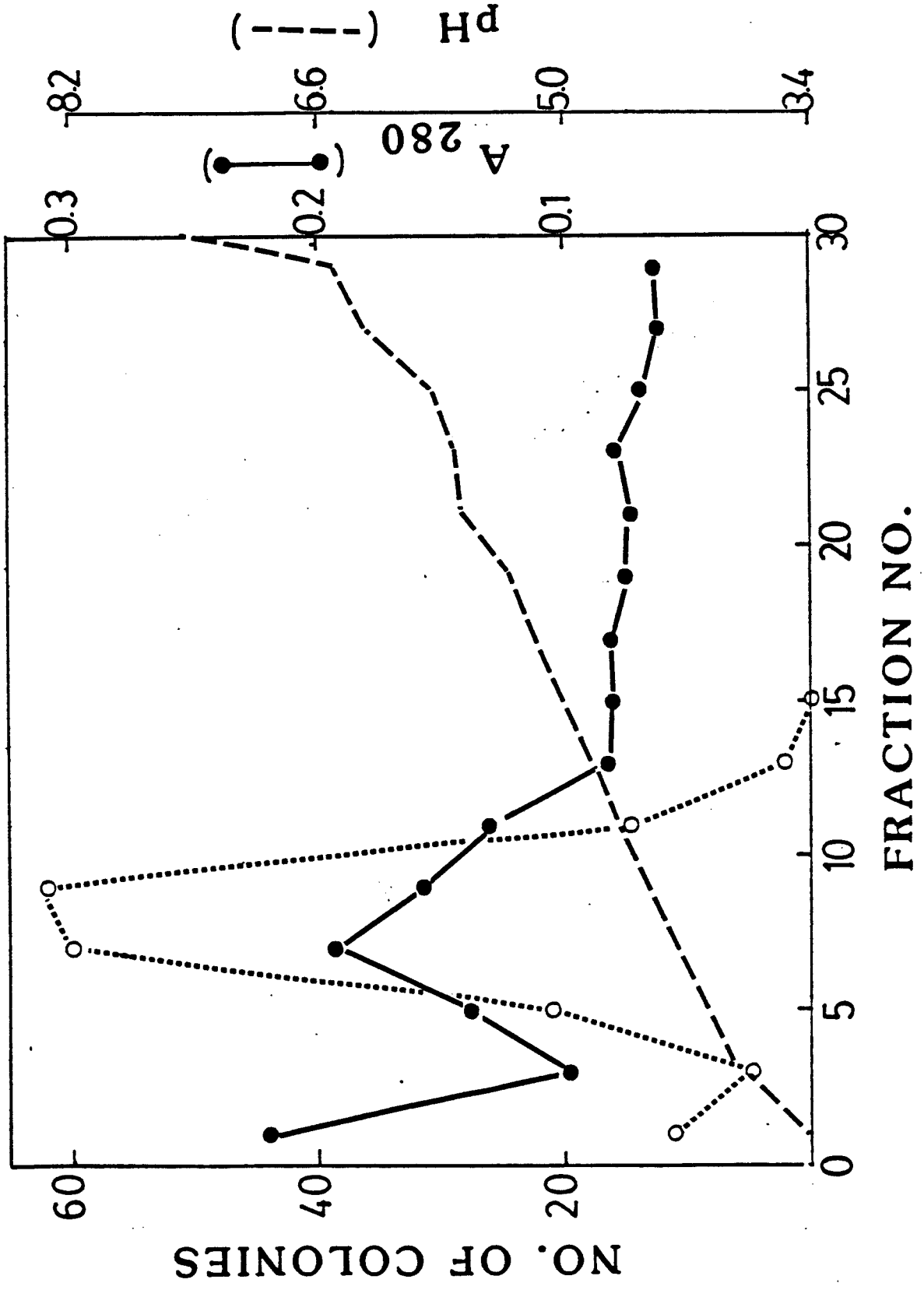
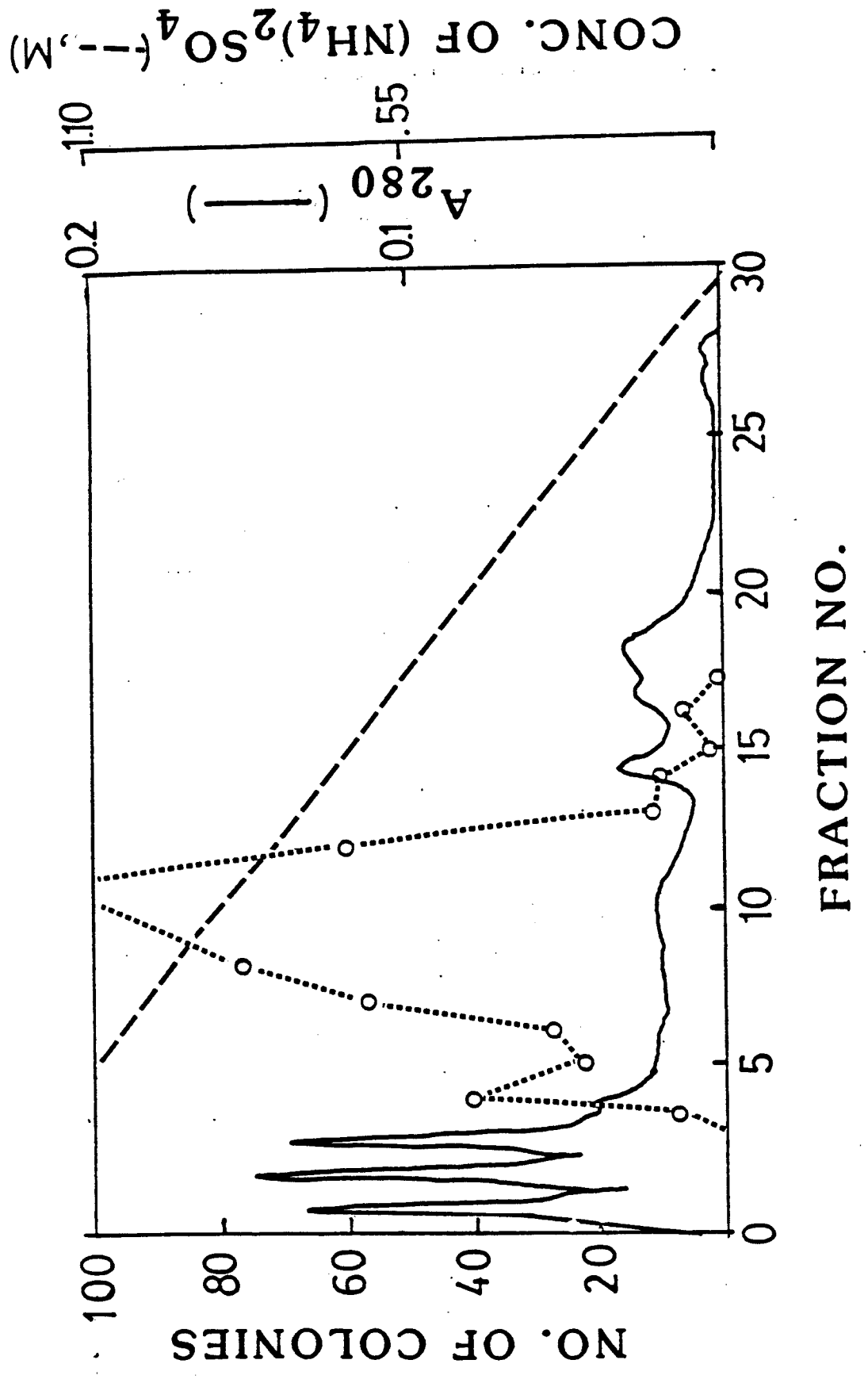

NO. OF COLONIES
60
40
20
A 280
0.3
0.2
0.1
pH
8.2
6.6
5.0
3.4
0
5
10
15
20
25
30
FRACTION NO.

Fig. 6 - Fractionation of UC-CSF by HPLC with TSK phenyl-5PW column. The fractions containing CSF activity from isoelectrofocusing (80 ml) were pooled, concentrated and injected onto an HPLC TSK phenyl-5PW column. Samples (2 ml) from eluted fractions were desalted by PD-10, sterilized by filtration and assayed for CSF activity. The highest CSF activity is 106 col colonies at fraction 11. Number of colonies is expressed by o---O .


NO. OF COLONIES
100
80
60
40
20
0
5
10
15
20
25
30
FRACTION NO.
$A_{280}$ (——)
0.2
0.1
1.10
.55
CONC. OF $(NH_4)_2SO_4$ (- - -, M)

activity in the growth medium as well as serum-free conditioned medium. Fig.7 shows a primary culture of human umbilical vein endothelial cells and Fig.8 shows endothelial cells continuous culture. Growth medium from the cultured cells was collected and pooled as above for further fractionation.

Growth medium from endothelial cell cultures was concentrated, dialyzed and applied to a DEAE-Sepharose column. The fractionation profile is shown in Fig.9. The CSF activity was eluted as a well resolved single peak at 0.13 M NaCl, very similar to the activity from UCCM that had 0.6 fold purification and 20.3% recovery. Both the break through fractions at 0.05 M NaCl and the high salt wash fractions at 0.5 M NaCl had no activity.

As shown in Fig.10, the CSF activity from endothelial cell cultures appeared at fractions 70-80 after Ultrogel ACA 44 chromatography. The Mr is estimated at 35,000 which is slightly higher than the CSF from UCCM. The purification was 1.05-fold and the recovery was 9.3%. Whether the CSF activities from these two sources are different can not be acertained until further characterization. The two-step purification of CSF from EC-CSF is summerized in Table II.

Characterization of CSF from UCCM and ECCM

CSF activity from UCCM or ECCM was partially purified by DEAE-Sepharose and gel filtration chromatography as above

Fig. 7 - Primary culture of human endothelial cells. Human endothelial cells obtained from umbilical cord as described in the Methods were plated in complete culture media in a 60 x 15 mm dish. 24 hr after the initiation of the culture, the cells were photographed by inverted phase contrast microscopy at 400X magnification.

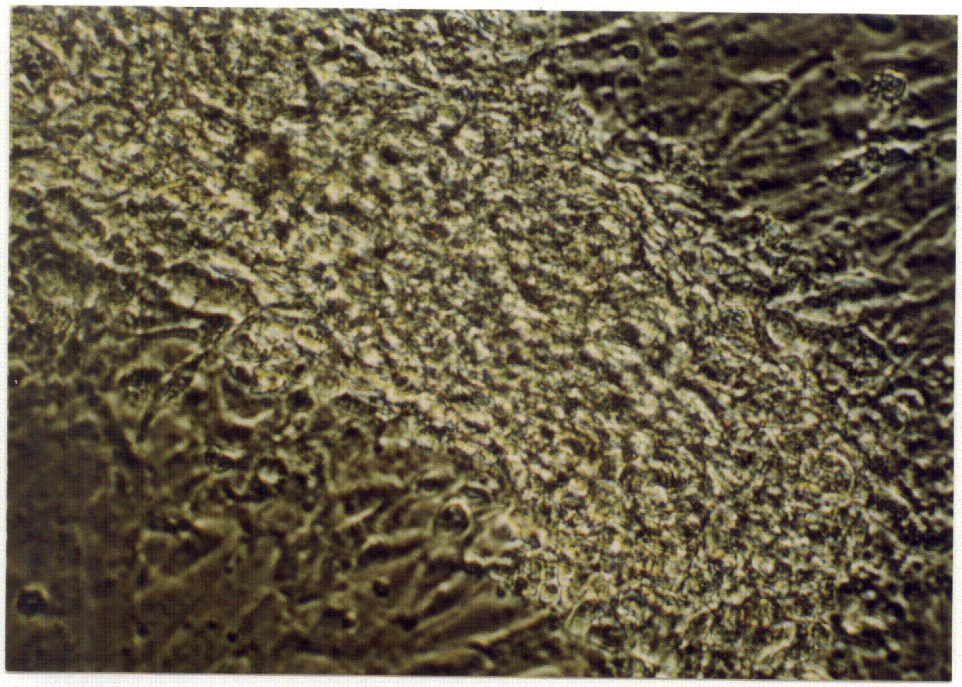


Fig. 8 - Human umbilical endothelial cells in continuous culture. Conditions are as described in the legend to Figure 7. The cells were 72 hr after the initiation of the culture.

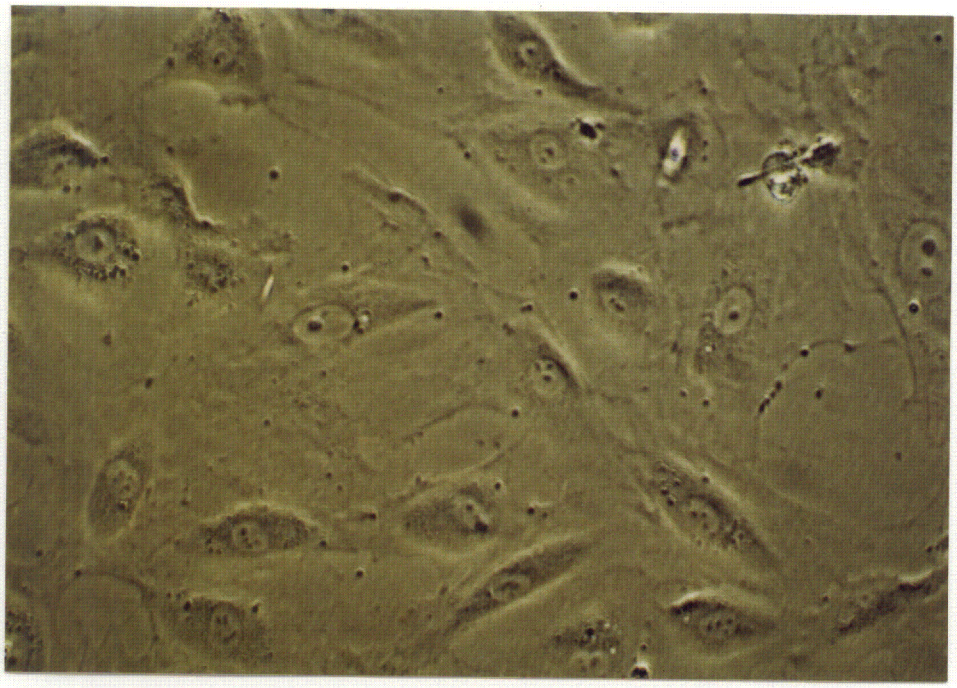


Fig. 9 - Fractionation of EC-CSF on a DEAE Sepharose CL-6B column. Conditions are as described in the legend to Figure 2. The ECCM prepared from cultures was concentrated to 43 ml, then loaded onto DEAE Sepharose column.

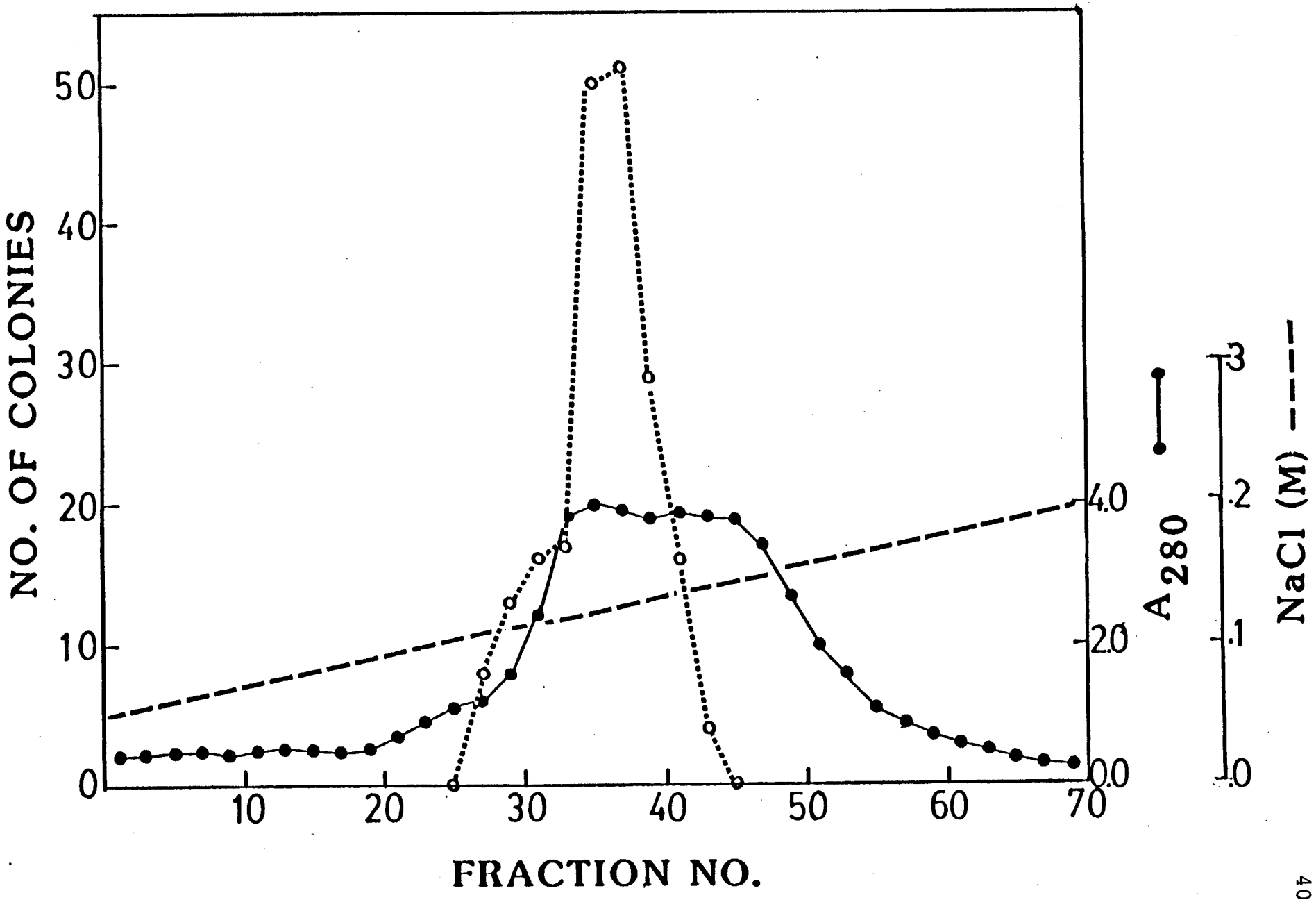

NO. OF COLONIES
50
40
30
20
10
0
10
20
30
40
50
60
70
FRACTION NO.
4.0
2.0
0.0
A280
.3
.2
.1
.0
NaCl (M) ----

Fig. 10 - Fractionation of EC-CSF on a ACA44 gel filtration column. Conditions are as described in the legend to Figure 3. Active fractions from the DEAE column (140 ml) were pooled and processed.

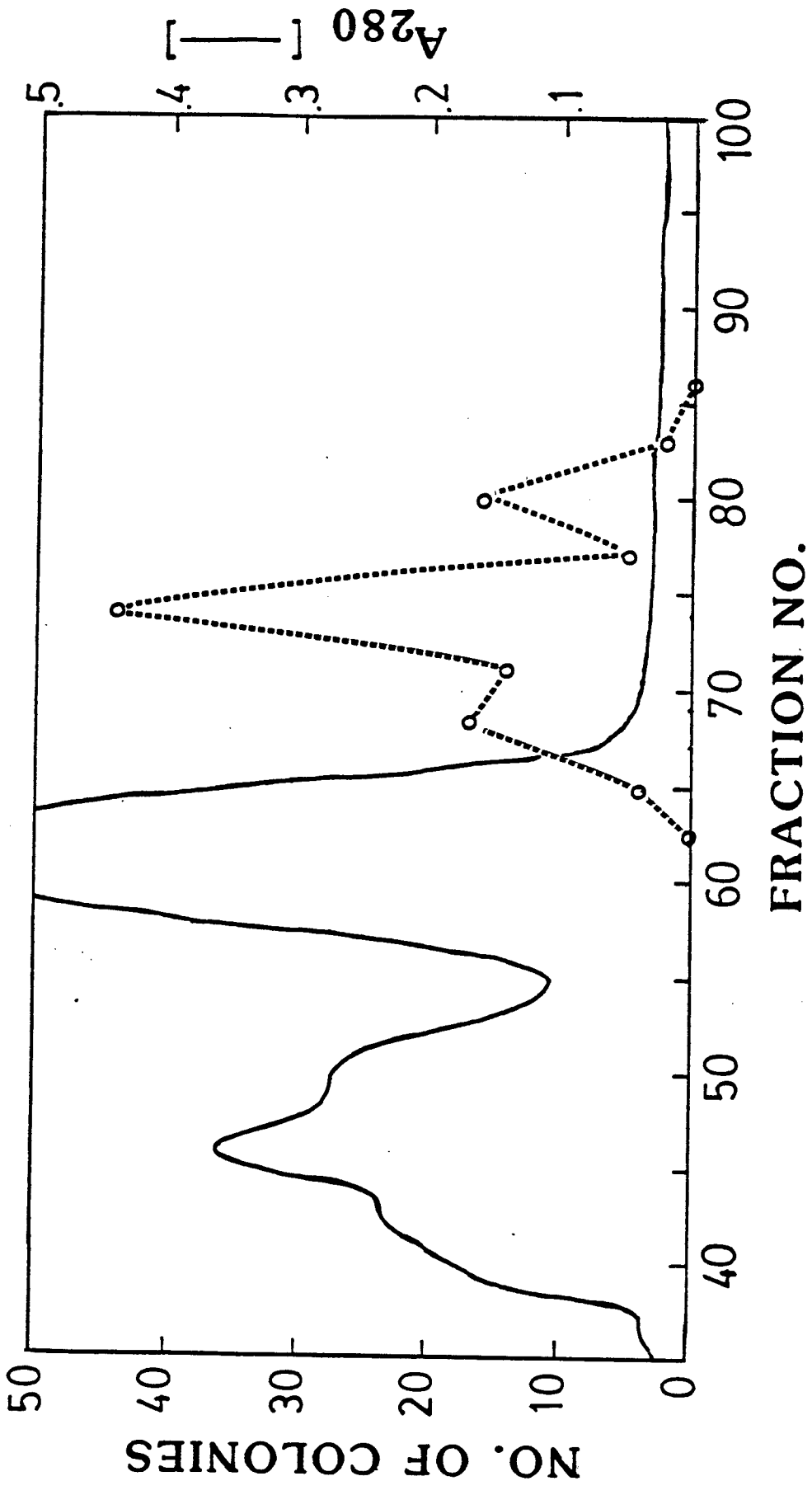

$A_{280}$ [——]
5.
4.
3.
2.
1
100
90
80
70
60
50
40
FRACTION NO.
NO. OF COLONIES
50
40
30
20
10
0

TABLE II

FRACTIONATION OF ENDOTHELIAL CELL-COLONY STIMULATING FACTOR

| STEP | PROTEIN (mg) | TOTAL ACTIVITY (units) | SPECIFIC ACTIVITY (units/mg) | PURIFICATION (fold) | YIELD (%) |
|---|---|---|---|---|---|
| CONDITIONED MEDIA | 918 | 752,000 | 819 | 1.00 | 100 |
| DEAE-SEPHAROSE | 315 | 153,000 | 486 | 0.59 | 20 |
| ACA 44 | 81 | 70,000 | 864 | 1.05 | 9 |

and was characterized as shown below.

Both EC-CSF and UC-CSF are stable at $60^0$C for 30 min. At $80^0$C or higher for 5 min, both CSF lose activity completely as shown in Table III. Both EC-CSF and UC-CSF are sensitive to the digestion by subtilisin, chymotrypsin and trypsin in that order (Table IV). The data indicate the proteinacious nature of these CSFs. In addition the EC-CSF appears to be more resistant to the protease treated than is UC-CSF.

The glycoprotein nature of CSF has been well documented (67). Although it is quite evident that carbohydrate side chains of CSF are not required for the biological activity (67), the CSF activity is always found to be sensitive to periodate oxidation. As shown in Table V, periodate at 5 and 10 mM has an inhibitory effect on both EC-CSF and UC-CSF to an equal extent. Incubation of dithiothreitol up to 10 mM concentration with EC-CSF and UC-CSF, results in about 50% incubation, Table VI. Thesis data suggest that disulfide bond might be important to the biological activity.

Regulation of CSFs Production in Cultured Endothelial Cells

To investigate whether the production of CSF from cultured endothelial cells requires DNA, RNA or protein synthesis, inhibitors of the above were used and the results are shown in Table VIII. Cycloheximide (100 ug/ml), puromycin (10 ug/ml) and actinomycin D (1 ug/ml) completely

TABLE III

TEMPERATURE STABILITY OF EC-CSF AND UC-CSF

| TEMPERATURE (°C) | EC-CSF (NO. OF COLONIES) | UC-CSF (NO. OF COLONIES) |
|---|---|---|
| 25 | 82 | 64 |
| 50 | 74 | 67 |
| 60 | 62 | 58 |
| 80 | 0 | 0 |
| 100 | 0 | 0 |

* All the data are from the average of duplicate samples and the deviation is less than 10%.

TABLE IV

PROTEASE DIGESTION OF EC-CSF AND UC-CSF

| TREATMENT | EC-CSF (NO. OF COLONIES) | UC-CSF (NO. OF COLONIES) |
|---|---|---|
| CONTROL | 141 | 89 |
| TRYPSIN | 41 | 11 |
| CHYMOTRYPSIN | 22 | 3 |
| SUBTILISIN | 0 | 0 |

* All the data are from the average of duplicate samples and the deviation is less than 10%.

TABLE V

PERIODATE OXIDATION OF EC-CSF AND UC-CSF

| PERIODATE (mM) | EC-CSF (NO. OF COLONIES) | UC-CSF (NO. OF COLONIES) |
|---|---|---|
| 0 | 103 | 87 |
| 1 | 112 | 84 |
| 5 | 88 | 65 |
| 10 | 4 | 16 |

* All the data are from the average of duplicate samples and the deviation is less than 10%.

TABLE VI

EFFECT OF SULFHYDRYL REAGENT ON COLONY FORMATION

| DITHIOTHREITOL(mM) | EC-CSF (NO. OF COLONIES) | UC-CSF (NO. OF COLONIES) |
|---|---|---|
| 0 | 100 | 87 |
| 1 | 73 | 76 |
| 5 | 42 | 53 |
| 10 | 40 | 50 |

* All the data are from the average of duplicate samples and the deviation is less than 10%.

TABLE VIII

EFFECT OF INHIBITORS OF MACROMOLECULAR ON CSF BIOSYNTHESIS IN CULTURED ENDOTHELIAL CELLS

| | 0ug/ml | 0.5ug/ml | 1ug/ml | 5ug/ml | 10ug/ml | 100ug/ml |
|---|---|---|---|---|---|---|
| ARA C | 84 ± 4 | - | 85 ± 3 | 69 ± 13 | - | - |
| ACT D | 84 ± 4 | 4 ± 1 | 0 ± 0 | - | - | - |
| CYCLOHEXIMIDE | 113 ± 16 | - | 70 ± 9 | - | 63 ± 7 | 0 ± 0 |
| PUROMYCIN | 113 ± 16 | - | 15 ± 2 | - | 1 ± 1 | 0 ± 0 |

- Not determined.

* Data expressed as the mean ± SD.
n=3

inhibited the production of CSF, indicating that protein and RNA synthesis are obligatory for CSF production. Cytosine arabinoside, the DNA synthesis inhibitor, did not inhibit CSF production even at a concentration of 5 ug/ml, suggesting DNA synthesis (or cell proliferation) is not required for CSF production. This observation is consistent with confluent endothelial cells in serum-free M199 medium continuing to produce CSF activity for more than one week.

Several mitogens were used to examine their effects on CSF production and the results are shown in Table VII. Lipopolysaccharide stimulates CSF production 2-fold and PHA also stimulate slightly. However, both Con A and Pokeweed mitogen decreased in CSF production, probably due to cell aggregation in the presence of theses lectins. The LPS stimulation of CSF production is consistent with many previous reports (44,65). In order to rule out direct mitogen stimulation of the bone marrow cell to cause colony formation, 10 ug/ml of each mitogen were added to the assay without the addition of CSF. No colony formation was observed in any of the above assays.

The mechanism of regulation of CSF production has not been studied. Since cyclic nucleotides represent a prominent class of second messengers in the regulation of cellular metabolism, their effect on CSF production by endothelial cells in culture was examined. As shown in Table IX, dibutyryl cAMP and isobutylmethylxanthine, a

TABLE VII

EFFECT OF MITOGENS ON CSF PRODUCTION IN CULTURED ENDOTHELIAL CELLS

| MITOGEN | NO. OF COLONIES |
|---|---|
| CONTROL | 84 ± 4 |
| POKEWEED | 60 ± 4 |
| PHYTOHEMAGGLUTININ | 98 ± 4 |
| CONCANAVALIN A | 65 ± 1 |
| LIPOPOLYSACCHARIDE | 163 ± 1 |

* The data expressed as the mean ± SD.

n=3

TABLE IX

EFFECT OF CYCLIC NUCLEOTIDES ON CSF PRODUCTION IN CULTURED ENDOTHELIAL CELLS

| | 0 M | $10^{-7}$M | $10^{-8}$M | $10^{-9}$M |
|---|---|---|---|---|
| DIBUTYRYL cAMP | 82 ± 13 | 58 ± 20 | 51 ± 7 | 71 ± 6 |
| DIBUTYRYL cGMP | 82 ± 13 | 74 ± 5 | 74 ± 2 | - |
| ISOBUTYLMETHYL-XANTHINE | 82 ± 13 | 54 ± 9 | 67 ± 16 | 80 ± 18 |

* Data expressed as the mean ± SD.

n=3

phosphodiesterase inhibitor, produced a 40% inhibition in CSF production at concentration of $10^{-7}$ to $10^{-8}$ M. Dibutyryl cGMP, on the other hand, did not show any significant effect. Arachidonic acid metabolites represent another important classs of regulators of cellular metabolism. Table X shows the effect of prostaglandin $E_2$ and $F_2$ on CSF production. The results suggest that $PGE_2$ inhibits CSF production (40%) at concentrations as low as $10^{-9}$ M while $PGF_2$ stimulates production by 50% at a concentration of $10^{-6}$ M.

As shown in Table XI, lactoferrin at a concentration of $10^{-6}$ M inhibits CSF production by about 70%. Even at a concentration of $10^{-9}$ M, it still showed appreciable degree of inhibition (40%). Transferrin, however, gives a very small inhibitory effect, if any.

TABLE X

THE EFFECT OF THE ARACHIDONIC ACID METABOLITES ON CSF PRODUCTION IN CULTURED ENDOTHELIAL CELLS

| | 0 M | $10^{-6}$M | $10^{-8}$M | $10^{-9}$M |
|---|---|---|---|---|
| $PGE_2$ | 82 ± 13 | 50 ± 8 | 49 ± 4 | 52 ± 6 |
| $PGF_2$ | 82 ± 13 | 121 ± 13 | - | - |

- Not determined.

* Data expressed as the mean ± SD (n=3) of colony number per assay.

TABLE XI

THE EFFECT OF THE IRON PROTEINS ON CSF PRODUCTION IN CULTURED ENDOTHELIAL CELLS

| CONC. (M) | LACTOFERRIN | | TRANSFERRIN | |
|---|---|---|---|---|
| | NO. OF COLONIES | % ACTIVITY | NO. OF COLONIES | % OF ACTIVITY |
| 0 | 78 ± 6 | 100 | 78 ± 6 | 100 |
| $10^{-9}$ | 52 ± 8 | 67 | 69 ± 4 | 88 |
| $10^{-8}$ | 44 ± 5 | 56 | 68 ± 12 | 87 |
| $10^{-7}$ | 44 ± 3 | 56 | 70 ± 4 | 90 |
| $10^{-6}$ | 21 ± 4 | 27 | 65 ± 13 | 83 |

* Data expressed as the mean ± SD.

n=3

## Discussion

Results presented in this study have shown that conditioned media from human umbilical cord segments and endothelial cell cultures contain colony-stimulating factor activity. A five-step purification procedure involving column chromatography on DEAE-Sepharose, ACA44 gel filtration, Con A Sepharose, isoelectrofocusing and HPLC-TSK phenyl has been used to purify CSF from umbilical cord conditioned medium (UCCM). Although over 100-fold purification has been achieved with a final specific activity of 35,000 units/mg protein, the overall yield of 1% recovery is very poor. Similarly, the purification of CSF from cultured endothelial cells has also encountered difficulties as indicated in Table II. The most serious problems in purifying CSF from the UCCM or ECCM are the high protein content in the crude preparation and the instability of CSF during the purification. Similar problems also existed in the early purification of CSF from human lung- and placenta- conditioned media. The reason for the loss of CSF activity during the purification is not clear, however, nonspecific protein aggregation and protease digestion may account for some of the losses. It is clear from this study that neither UCCM nor ECCM is a good source for the purification of CSF although the endothelial cell in culture

provides a model system to study the regulation of CSF biosynthesis. Although the question of the type of CSF produced cannot be answered conclusively, the physical, chemical and biological properties such as molecular weight, isoelectric point, suggest that a GM-CSF is produced. A previous report by Irvine et al (46) has indicated that the main source of the CSF in the umbilical cord is found in the Wharton's Jelly component and the morphological and cytochemical analysis of the colonies formed indicate they are composed of over 98% granulocytic cells, thus indicating a G-CSF in nature. However, in this experiment, the conditioned medium prepared from the umbilical cord segments after the removal of vein endothelial cels by collagenase treatment did not contain and CSF activity, suggesting that vein endothelial cells are probably the major CSF-producing component (data not shown). Further biochemical characterization of CSF from different components of the umbilical cord is needed to resolve this discrepancy. The properties of CSF activity produced by cultured endothelial cells are very similar to those described in a previous report (47). Ascensao et al . (47) have described a GM-CSF partially purified from the conditioned medium of human umbilical vein endothelial cells in culture. However, they were also able to detect a multi-CSF activty with an Mr of 30,000 and pI values of 4.5 and 7.2 and a GM-CSF activiy with a Mr of 15,000. The low Mr GM-CSF has also sown

activity on BFU-E. It is possible that endothelial cells produce other hematopoietic factors other than GM-CSF. A recent report by Adamson et al . (66) further indicates that GM-CSF is produced by endothelial cells that have been stimulated by tumor necrosis factor.

Regulation of CSF production has been studied by several groups. Quesenberry and Gimbrone (65) have reported that endotoxin and granulocytes stimulated CSF production by primary cultures of human vascular endothelial cells. Their observations suggested that the vascular endothelium might play a role in the physiologic regulation of granulopoiesis. In this study, bacterial lipopolysaccharides and phytohemagglutinin have shown stimulated CSF production (Table VII). The inhibitory effects of actinomycin D and cycloheximide or puromycin in the culture showed that RNA and protein synthesis are required for CSF production (Table VIII). Recently, Bagby et al .(62) have reported that monocyte-secreted proteins (monokines) regulate CSF production by vascular endothelial cells. Although lactoferrin inhibited monokine production, it does not inhibit CSF production by monokine-stimulated endothelial cells. The stimulation of CSF production by monokines has not been tested in this study. However, lactoferrin, but not transferrin, was shown to inhibit CSF production in cultured umbilical endothelial cells (Table XI). The nature of the monokines involved in the regulation of GM-CSF production is

not known at present. However, both interleukin 1 and tumor necrosis factor (TNF) have shown their effect on GM-CSF production both in endothelial cells (64) and lung fibroblasts (63). Thus, Zucali et al . (63) have reported the stimulation of GM-CSF and prostaglandin E production by lung fibroblasts when stimulated by recombinant human IL-1. Munker et al (64) have described the stimulation of GM-CSF production in lung fibroblasts and vascular endothelial cells by TNF. Northern blot analysis clearly indicated the increase of GM-CSF mRNA and the mechanism of action is probably at the transcriptional control level.

In order to investigate the regulatory mechanism of CSF production, the effect of most common metabolic regulators such as cAMP, cGMP, PGE and PGF were tested in this study. Cyclic nucleotides usually mediate the hormonal regulation of various cellular functions through a cyclic nucleotide-dependent phosphorylation mechanism. Attention is currently being focused on the possible role of cyclic nucleotides and the major enzymes of cyclic nucleotide metabolism in the regulation of endothelial cell function. In addition to cyclic nucleotides, prostaglandins derived from essential fatty acid via the unsaturated fatty acids arachidonic acid have varied physiologic effects. They have been implicated in the contraction of smooth muscle, aggregation of platelets and inflammation. Preliminary results have shown that cAMP and $PGE_2$ inhibit CSF

production while $PGF_2$ enhances its production. The molecular mechanism of action remains to be studied. Since the action of monokines in modulating CSF production is likely to be a receptor-mediated mechanism, a study of receptor binding by monokines in endothelial cells and the subsequent metabolic events resulting in stimulation of the expression of the GM-CSF gene will be carried out in the near future.

Studies utilizing human umbilical vein derived endothelial cells have now clearly shown that in short term culture they are capable of producing colony-stimulating factor activity against both human and mouse marrow cells. Further studies revealed that CSF from human umbilical vein endothelial cells in culture can be regulated by several regulators. AS noted above, umbilical vein derived endothelial cell is a potential attractive candidate regulator cell for granulopoiesis. However, at present the bulk of evidence suggests that CSF is probably a physiologic regulator of granulocyte, macrophage production and that endothelial cells may constitute an important in vivo source for this regulator.

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