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Effect of Colony-stimulating Factors on the Proteins Synthesized by Normal and Leukemic Myeloid Progenitor Cells*

A. W. Burgess, P. C. Cooper, I. J. Stanley, and N. A. Nicola

A. Introduction

The proliferation and differentiation of normal granulocyte-macrophage (GM) progenitor cells is dependent on the presence of GM colony stimulating factor (GM-CSF). The proliferation of primary leukemic cells is also dependent on GM-CSF, and some established myelomonocytic cell lines (e.g., WEHI3B D^+) can be induced to differentiate by G-CSF.

Using fluorescence-activated cell sorting, it has been possible to isolate highly purified granulocyte-macrophage colony forming cells (GM-CFC) [11]. Fetal liver cells can be labeled with fluorescent pokeweed mitogen and the GM-CFC sorted on the basis of fluorescence intensity and light scatter. We have initiated an analysis of the ^{35}S-labeled proteins in GM-CFC using two-dimensional gel electrophoresis [12]. In particular, the protein changes induced in GM-CFC by macrophage colony stimulating factor (M-CSF) [14], GM-CSF [4], and other hemopoietic regulators [5] have been monitored in an attempt to understand the extent and function of the protein synthetic events associated with commitment to differentiation or proliferation.

Our laboratory has available a murine leukemic cell line [WEHI3B (D^+)] [9] with many similar properties to GM-CFC. A subspecies of CSF (G-CSF), present in the serum of mice treated with endotoxin [3], is able to stimulate WEHI3B (D^+) cells to differentiate to form relatively mature myeloid and monocytic cells. Electrophoretic analysis of extracts from WEHI3B (D^+) cells stimulated by G-CSF has been used to compare the ^{35}S-labeled proteins of these leukemic cells with mature myeloid cells and GM-CFC.

B. Materials and Methods

I. Cells and Culturing

Normal colony-forming cells were purified from murine fetal liver (13–14 day gestation) as described previously [11]. They showed a colony-forming efficiency of 26% and a clone-forming efficiency of 70%–90%.

WEHI3B (D^+) cells were established as a cloned cell line from a mineral oil induced myelomonocytic leukemia (WEHI-3) [15]. Cells were biosynthetically radiolabeled using ^{35}S-methionine (3.7×10^{10} Bq/mmol) (Amersham), at 9.3×10^6 Bq/ml for 3 h at 37 °C in methionine-depleted DME containing FCS (5% v/v) supplemented with methionine (1.3×10^{-5} *M* final concentration). The cells were grown to a density of 10^6/ml for radiolabeling, and after labeling were washed three times with mouse tonicity phosphate buffered saline (MTPBS) before preparation for electrophoresis or nuclear extraction. Bone marrow neutrophils

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were prepared by cell sorting as described previously [16].

II. Preparation of Stimulating Factors

G-CSF was partially purified from ES (50 ml) using gel filtration as described previously [3]. The partially purified preparation of G-CSF was used at 2% v/v final concentration in the experiments to be described. This concentration was a supramaximal stimulus for induction of WEHI3B (D^+) differentiation. Pokeweed mitogen stimulated spleen-conditioned medium [13], GM-CSF from mouse lung conditioned medium [4], and M-CSF from pregnant mouse uterus extract [2] were prepared as described previously.

III. Preparation of Nuclei

Cells were placed into an ice cold hypotonic Tris-HCl buffer (10 m*M* Tris, pH 7.4, 3 m*M* $MgCl_2$) for 5 min (10^7 cells/ml), and lysed using a Dounce glass homogenizer (type B). The disrupted cells were carefully layered over a sucrose solution (0.65 *M* sucrose, 10 m*M* Tris pH 7.4, .3 m*M* $MgCl_2$) and centrifuged at 6000×g for 10 min. Nuclear pellets were resuspended in the hypotonic solution and recentrifuged through 0.65 *M* sucrose.

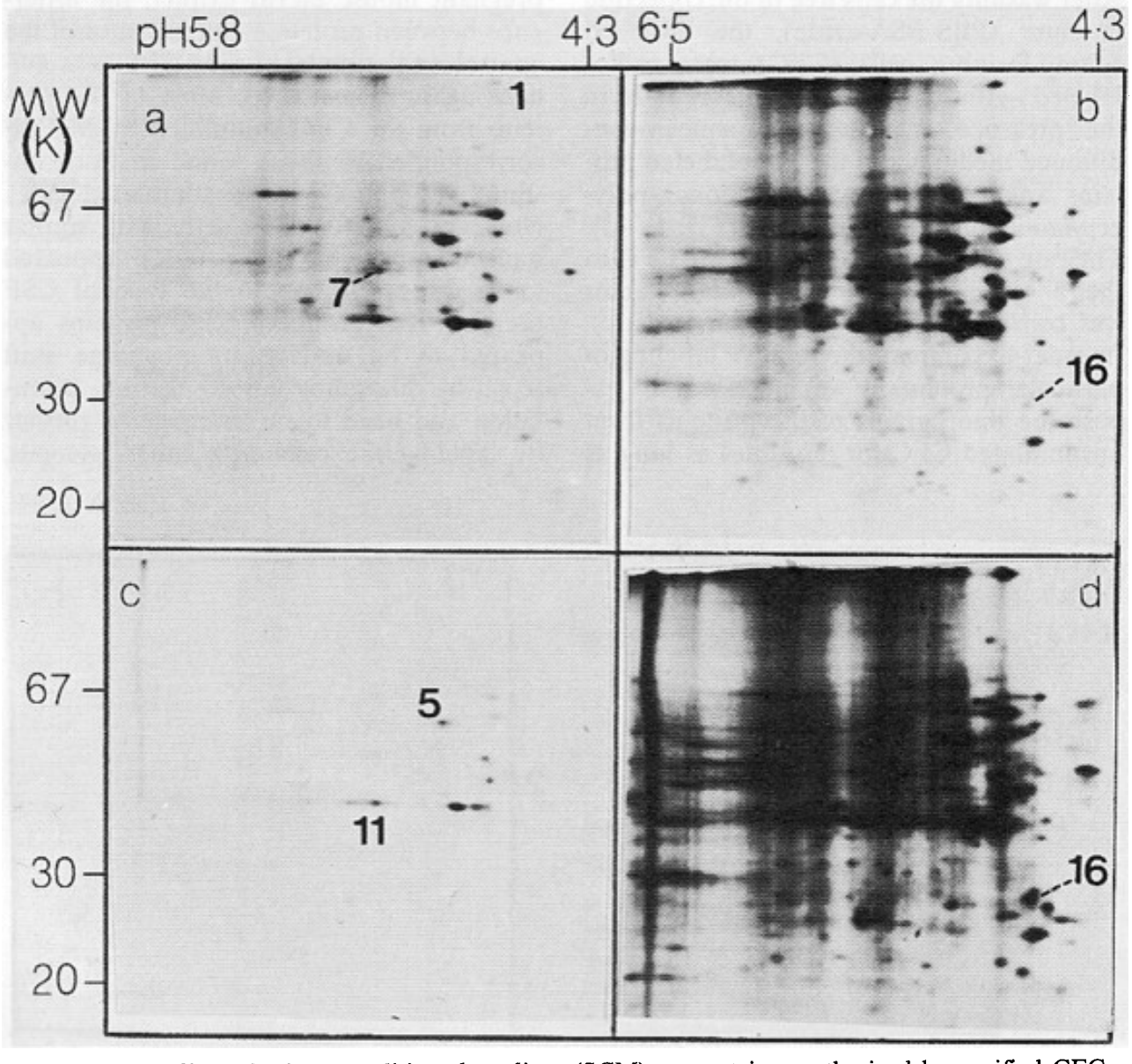

Fig. 1a–d. Effect of spleen-conditioned medium (SCM) on proteins synthesized by purified CFC. **a** Incubation for 18 h with SCM and ^{35}S-methionine (200 μCi/ml); the gel (12%) was processed for fluorography and exposed for 5 h. **b** As for **a** exposed for 20 h. **c** Incubated for 18 h without stimulus in the presence of ^{35}S-methionine (200 μCi/ml) exposed for 20 h. **d** As for **c** exposed for 20 days

IV. Electrophoretic Analysis of ^{35}S-Labeled Proteins

Samples were prepared for two-dimensional electrophoretic analysis by the method of O'Farrell [12]. Radiolabeled proteins from the cells were analyzed using SDS polyacrylamide gel electrophoresis (10% acrylamide cross-linked with 0.25% bisacrylamide) by the Laemmli and Favre [7] procedure. Radiolabeled proteins were detected by fluorography [1].

C. Results and Discussion

I. Protein Synthesis in Colony-Forming Cells

After washing the cells free of the collection medium (PBS-BSA-azide), the enriched colony-forming cells (CFC) were radiolabeled with ^{35}S-methionine for 18 h in the presence or absence of spleen-conditioned medium and the radiolabeled proteins analyzed by two-dimensional polyacrylamide gel electrophoresis (Fig. 1 a–d). The rate of protein synthesis by the CFC in the absence of colony-stimulating factor was considerably reduced (cf., Figs. 1 a, c). In order to compare the relative labeling of particular proteins it was necessary to expose the fluorograms of the proteins from unstimulated CFC for ten times as long as the fluorograms of the proteins from CFC labeled in the presence of CSF. Prolonged exposure of the fluorograms (20 h for the proteins from stimulated CFC and 20 days for the proteins from unstimulated CFC) indicated that most of the proteins in the surviving CFC were synthesized in the same proportion in the presence or absence of CSF (cf., Figs. 1 b, d). However, the small group of proteins near pI 5.5 and molecular weight 23–25K were evident in the unstimulated CFC but not in the proliferating CFC. Similar results were obtained when purified GM-CSF from mouse lung conditioned medium was used to stimulate the CFC.

When the GM-CFC were incubated with another form of CSF, i.e., M-CSF from pregnant mouse uterus extract, the difference between protein synthetic rates of the control and stimulated GM-CFC was evident again (Figs. 2 a, b). Most of the proteins from the CFC stimulated by M-CSF corresponded to those found in both unstimulated and GM-CSF stimulated CFC. However, a chain of proteins with similar molecular weight (near 69K) appeared to be quite sensitive to the type of CSF present. This group of CFC proteins appeared to be undergoing a charge shift (e.g., by phosphorylation) during stimulation and need to be investigated further by labeling the cells with other isotopes,

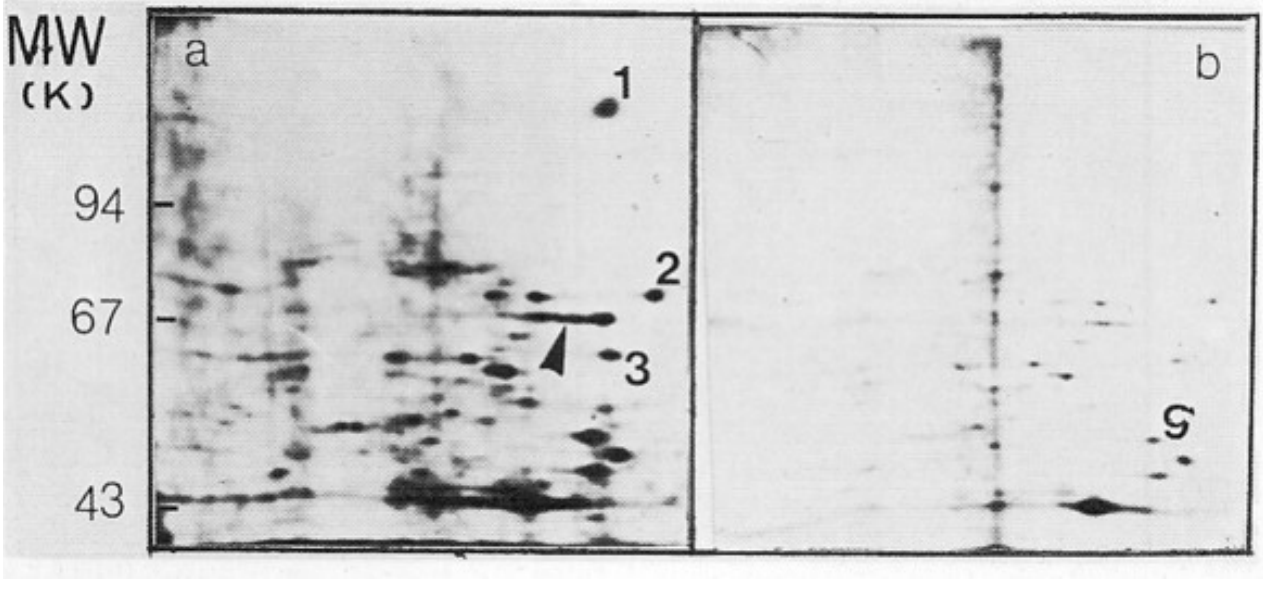

Fig. 2 a, b. Effect of pregnant mouse uterus extract (PMU-E) on proteins synthesized by purified CFC. **a** Incubation for 18 h with PMU-E and ^{35}S-methionine (200 μCi/ml); the gel (8%) was processed for fluorography and exposed for 2 days at – 80 °C. **b** Incubation for 18 h with medium and ^{35}S-methionine and the fluorograph exposed for 5 days

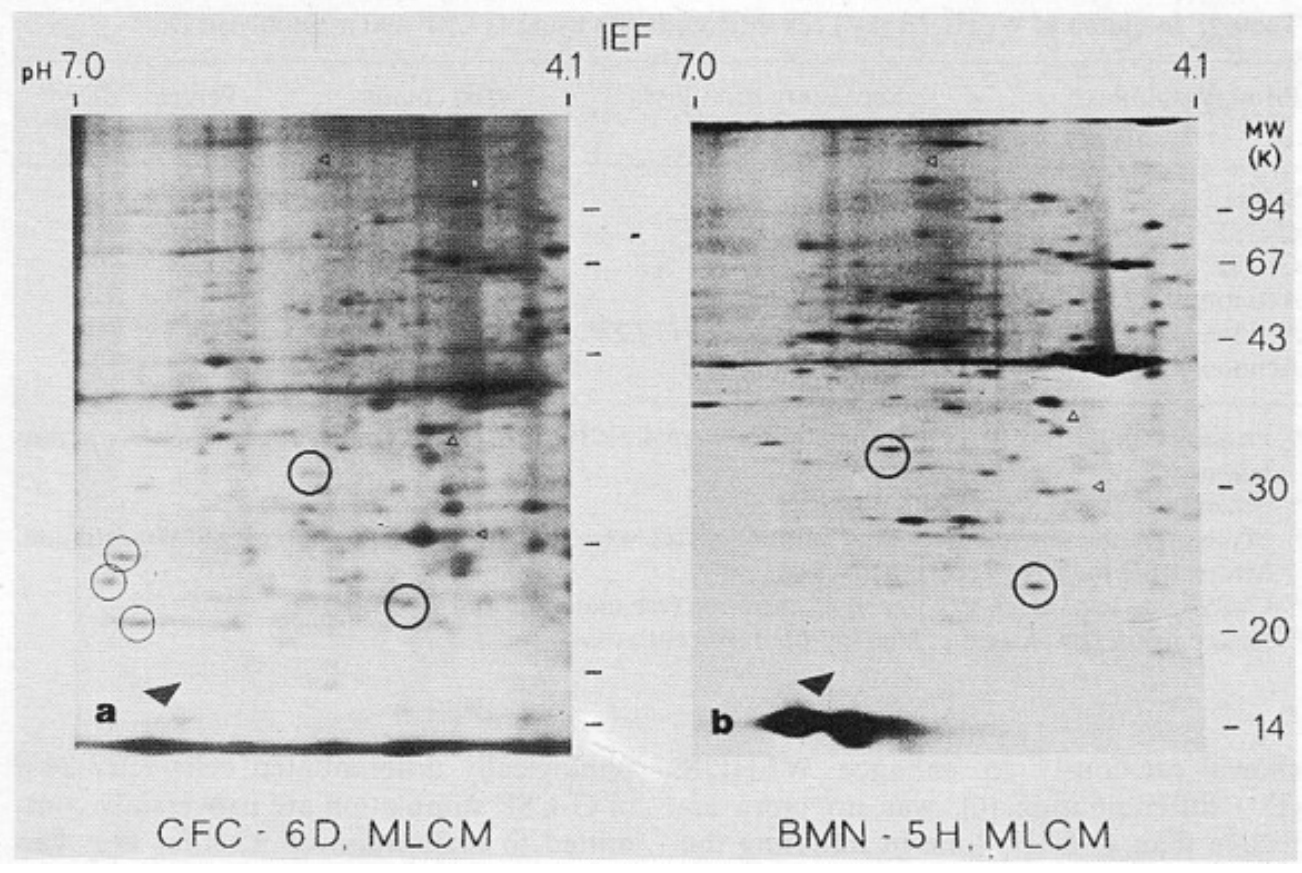

Fig. 3a,b. Comparison of two-dimensional electrophoretic profiles of ^{35}S-labeled proteins from **a** neutrophils produced in vitro by stimulating colony forming cells (CFC) using purified GM-CSF from mouse lung conditioned medium for 6 days and **b** bone marrow derived neutrophils stimulated for 5 h with GM-CSF. Some positions on the profiles are highlighted to allow the proteins to be compared more easily

e.g., $^{32}PO_4$. Again there are remarkably few changes apparent in the relative distribution of protein synthesis in the presence and absence of CSF, especially when previous reports on the protein changes induced by GM-CSF in the M1 leukemic cell line are considered [8].

II. In Vitro and Bone Marrow Derived Neutrophils

When the CFC were allowed to proliferate for 6 days in the presence of GM-CSF from mouse lung conditioned medium, normal myeloid differentiation occurred. The two-dimensional electrophoretic profiles of the ^{35}S-labeled proteins from these in vitro derived neutrophils were compared to a similar profile of ^{35}S-labeled proteins from neutrophils purified from bone marrow (Fig. 3a, b). As expected many of the proteins synthesized by bone marrow neutrophils were also synthesized by the neutrophils derived in vitro. Interestingly the proteins of the bone marrow derived neutrophil induced by GM-CSF were also present in the in vitro derived neutrophils. However, two low molecular weight proteins (approx. 14K) near pH 6 which were rapidly synthesized by bone marrow neutrophils had a very low rate of synthesis in the in vitro derived neutrophils.

III. Myelomonocytic Leukemic Cell Differentiation WEHI3B (D^+)

WEHI3B cells were cultured for 3 h in the presence of G-CSF factors or actinomycin D before being transferred to secondary liquid cultures in the presence or absence of G-CSF or actinomycin D for 24 h. The results of three experiments are summarized in Table 1. Under all culture conditions there was a threefold increase in cell numbers after the initial culture period. When WEHI3B (D^+) cells were cultured initially with G-CSF and then transferred to a culture without G-CSF, myelocytes and promonocytes were still produced (Table 1). Actinomycin D, which has been

Table 1. Initiation of WEHI-3B (D^+) cell differentiation using G-CSF and actinomycin D

Primary stimulus[a]	Secondary stimulus[b]	Cell counts	Percent differentiating cells[c]
Saline	Saline	1.6×10^7	6 ± 3
G-CSF[d]	Saline	1.6×10^7	26 ± 4
G-CSF	G-CSF	1.5×10^7	50 ± 5
Actinomycin D[e] + G-CSF	Saline	1.4×10^7	28 ± 3
Actinomycin D + G-CSF	Actinomycin D + G-CSF	1.3×10^7	68 ± 7
Actinomycin D	Saline	1.5×10^7	29 ± 4

[a] Primary culture was of 3 h duration: there were 4.9×10^6 in 20 ml of culture medium before stimulation
[b] Secondary culture was of 24 h duration
[c] Myelocytes, metamyelocytes, and promonocytes were classed as cells undergoing differentiation. Means ± SD for three experiments
[d] G-CSF was used at 2% v/v final concentration (see materials and methods)
[e] Actinomycin D was used at 5 ng/ml final concentration

shown previously to enhance WEHI3B (D^+) differentiation [6], was no more effective than G-CSF alone in initiating the production of promonocytes or myelocytes (Table 1). The continued presence of either G-CSF or actinomycin D in the secondary culture resulted in a greater degree of WEHI3B (D^+) differentiation. For example, reculture of G-CSF treated cells into medium containing G-CSF resulted in the production of 50% maturing cells, indicating that not all of the cells capable of differentiation in response to G-CSF or actinomycin D were able to be induced within 3 h. Table 1 also shows that actinomycin D enhanced the differentiation when cocultured with G-CSF for the entire culture period (68% differentiation).

Cell cytotoxicity was not found during these experiments, although the actinomycin D marginally decreased the rate of WEHI3B cell proliferation during the culture period. The commitment experiments indicated that events important to cellular differentiation were occurring (in a proportion of cells) within 3 h of treatment. Other data (not shown) have indicated that more cells initiated the differentiation programme after 5 h of treatment with G-CSF than after 3 h of stimulation. Extended culture of the short-term (3 h) G-CSF stimulated cells indicated that many of the cells continued to proliferate, and by 5 days only 10% of the cells were differentiated to any extent. It is still not clear whether the morphologically differentiated cells after 24 h of G-CSF stimulation are irreversibly committed to maturation or whether they can revert to producing blast cells [10].

An examination of the protein changes occurring during the early phases of the differentiation process was performed using two-dimensional gel electrophoresis. WEHI3B (D^+) cells were treated with G-CSF for 2 h and then biosynthetically radiolabeled with ^{35}S-methionine in the presence of G-CSF for a further 3 h. Figure 4 compares fluorographs from untreated WEHI3B (D^+) (Fig. 4a) with the G-CSF treated cells (Fig. 4b). The numbers of the figures indicate the molecular weight of the proteins, which change significantly when the cells are stimulated by G-CSF (letters have been used when two proteins with similar molecular weights need to be distinguished. Six proteins were found to be more heavily radiolabeled after G-CSF treatment (proteins 16a, 18, 35, 66, 85, and a very large molecular weight protein, labeled v1, Fig. 4b). Seven proteins were found to be radiolabeled to a decreased extent (29, 32, 34, 35a, 36, 67, and 84). The decreases in the incorporation of ^{35}S-methionine were found predominantly in acidic proteins (i.e., pI less than 4.8): 29, 32, 34, 35a, 36, 67, and 87. A protein of 16,000 daltons (number 16a in Fig. 4b) exhibited a large increase in label incorporation (6.4 times the amount found in the untreated control cells) (Fig. 4a).

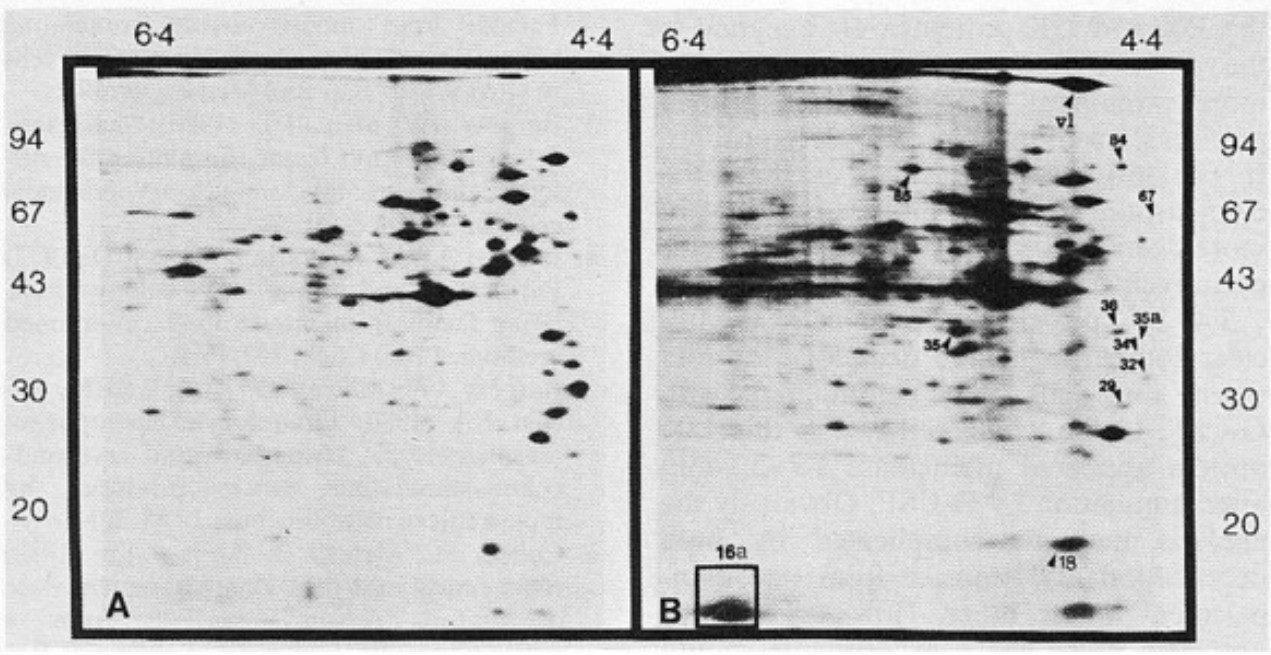

Fig. 4 A, B. WEHI3B cells were treated for 5 h with: **A** NS 2% v/v, **B** G-CSF 2% v/v, radiolabeled during the last 3 h of treatment and then subjected to two-dimensional gel electrophoresis and fluorography. *Arrows* indicate increases or decreases of protein synthesis, induced by G-CSF relative to the NS treated controls. *Numerals* associated with the *arrows* indicate molecular weight of proteins

Examination of the protein changes in the nucleus and cytoplasm of WEHI3B (D^+) at the initiation of differentiation were made using nonequilibrium pH gradient electrophoresis, isoelectric focusing, and SDS gel electrophoresis. The major protein synthetic changes after 5 h stimulation of WEHI3B (D^+) with G-CSF have been tabulated in Fig. 5. The 85K protein was not detected in the nucleus but was present in the whole cell lysates and has been assigned to the cytoplasm. Similarly

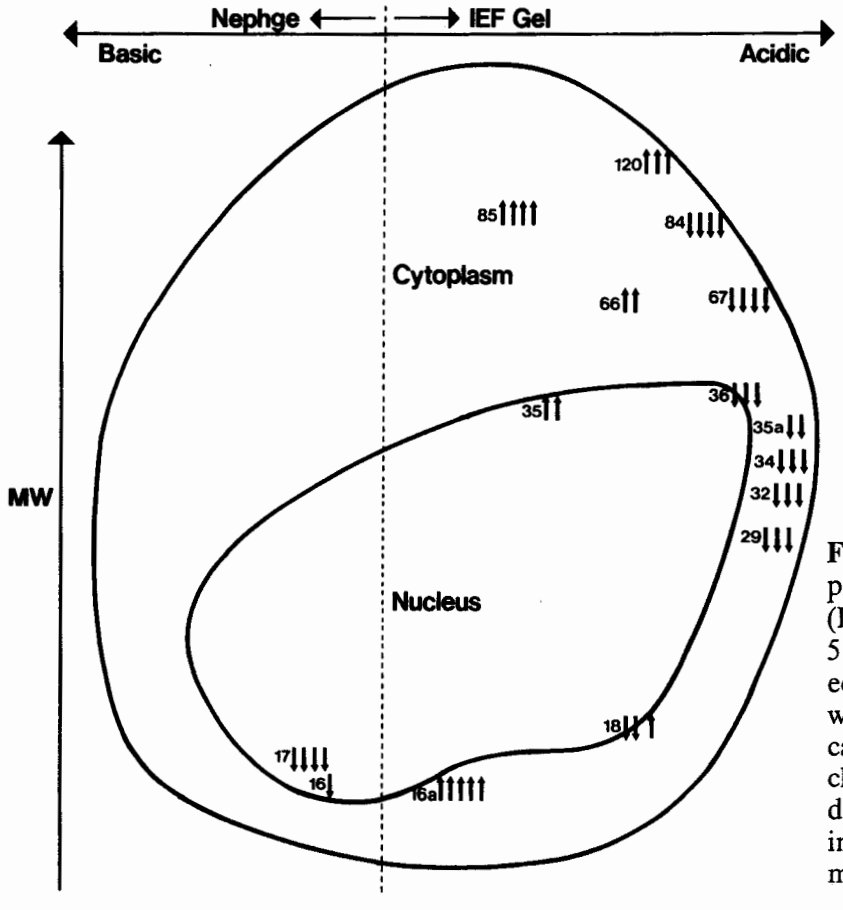

Fig. 5. Summary of changes in protein biosynthesis of WEHI3B (D^+) cells stimulated by G-CSF for 5 h. Subcellular location is indicated by the position of the *arrows* within the compartments. The location of the protein *arrows* on nuclear/cytoplasm boundaries indicates the presence of the protein in both the nuclear and cytoplasmic compartments

the 36K and 35K proteins were assigned to the nucleus because these proteins were more prominent on the nuclear protein profiles than the whole cell lysate profiles. It will still be necessary to examine the cytoplasmic and membrane proteins in more detail before assigning these proteins exclusively to the nuclear compartments.

The 18K protein was detected in the nuclear compartment and decreased 2.6-fold when the cells were stimulated with G-CSF. In the whole cell lysates this 18K protein appeared to increase 1.7–2.1-fold after stimulation by G-CSF. Obviously the analysis may be complicated by both degradation and transfer from one compartment to the other. However, the appropriate pulse chase experiments should be able to clarify the behavior of this protein.

D. Conclusion

Comparison of the protein profiles from the leukemic progenitor cells and normal progenitor cells indicated the presence of specific sets of proteins in common. Interestingly, less than 5 h stimulation of the WEHI3B (D^+) cells with G-CSF induced the production of some of the low molecular weight proteins characteristic of bone marrow neutrophils. Similarly, the synthesis of several of the proteins found in both the GM-CFC and the leukemic blasts was rapidly slowed down when G-CSF was added. Thus, the leukemic cells appear to be capable of an extremely rapid transition from the progenitor to mature cell compartment. Attempts are now being made to understand the function of the proteins common to the GM-CFC and WEHI3B (D^+) which decrease rapidly in response to G-CSF. In particular we are comparing these proteins to known viral oncogene products.

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