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Electrophoretic Analysis of the Cytoplasmic and Nuclear Protein Changes After Induction of Differentiation in WEHI-3B Myelomonocytic Leukemia Cells

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In response to a differentiation factor (G-CSF) the myelomonocytic leukemia cell line (WEHI-3B(D^+) differentiates to form mature macrophages and neutrophils. The effect of G-CSF on WEHI-3B(D^+) differentiation was augmented by low concentrations (5 ng/ml) of actinomycin D. Quantitative binding of an antineutrophil serum was used to segregate the differentiated cells from the leukemic blast cells. Molecular markers of later myeloid differentiation were detected in myelocytes and macrophages purified from differentiating WEHI-3B(D^+) cells. To study the initial molecular processes associated with the initiation of WEHI-3B(D^+) cells to differentiation, the protein changes were analyzed using gel electrophoresis. Quantitative analysis of the fluorographs from the two-dimensional (2D) electrophorograms of the ^{35}S-labeled proteins revealed major changes in the biosynthetic rates for 16 proteins within 5 hr: The biosynthesis of six proteins was increased and another ten proteins were synthesized at a reduced rate. Two of the proteins (17K and 36K daltons) were located in the nucleus. Pulse-chase experiments indicated that protein turnover for these proteins was rapid but the degradation of four proteins was suppressed. At least six of the proteins (16K to 120K daltons) were acidic and were associated with the cytoplasm. Electrophoretic analysis of the ^{35}S-labeled proteins indicated that a 35K protein induced by G-CSF was found in high abundance only in purified cells of intermediate differentiation (eg, myelocytes). Other proteins (eg, a very high molecular weight protein, and a 16K dalton protein) were obviously late markers of differentiated neutrophils or macrophages.

Key words: electrophoresis, NEPHGE, leukemia, differentiation, nuclear proteins, G-CSF, fluorescence-activated cell sorting

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It is possible to induce some myeloid leukemic cells to differentiate in vitro [1–4]. At least three murine leukemic cell lines have been extensively investigated: R-453 [1], M1(D+) [2], and WEHI-3BD+ [3], and one human promyeloblastic leukemia HL-60 [4]. All of these cell lines appear to differentiate in response to physiological regulators of hemopoiesis such as the colony-stimulating factors [5–9]. Initially it was thought that granulocyte macrophage colony-stimulating factor (GM-CSF [8], also called macrophage granulocyte inducer [5]) was the primary stimulus for the differentiation of the leukemic cells [10,11]. However, recent work has revealed that the differentiation factor (G-CSF) for leukemic cells can be separated from most of the GM-CSF usually present in unfractionated preparations of the stimulators [12–14]. Indeed, the differentiation factor appears to be a subspecies of CSF (G-CSF) which specifically stimulates granulocytic differentiation in normal cells [12,13].

Analysis of the molecular changes in WEHI-3B(D^+) cells stimulated by G-CSF offers the opportunity to study the switch from continuous proliferation (self-renewal) to the molecular changes which reflect the maturation of the leukemic cells toward neutrophils and macrophages. Previous studies using subclones of the M1 leukemia indicated rapid changes in hundreds of proteins after exposure to differentiation factor (G-CSF) [16,17]. Similar electrophoretic analyses of the initial protein changes in other cell lines capable of undergoing differentiation have, however, often shown many fewer changes, eg, myoblasts showed 30 protein changes [18], murine embryonic cells showed 36 changes on induction of development [19], and Friend erythroleukemia cells showed only 11 protein changes [20]. Rapid changes in a small number of proteins have also been reported after treatment of epithelial cells with hydrocortisone [21], fibroblasts with platelet-derived growth factor [22], and the human promyeloblastic leukemia HL-60 treated with dimethyl sulfoxide [23]. The discrepancy in the number of protein changes induced in M1 cells compared with other induction systems suggests that either disparate criteria have been used for the scoring of protein changes, or that there is an intrinsic difference between M1 cells and other leukemic cell systems (eg, perhaps related to the presence of C-type viruses in M1 cells) [24]. The WEHI-3B(D^+) myelomonocytic leukemia cell line appears to be an excellent model for biochemical comparison with the M1 cell line.

This manuscript demonstrates that reproducible changes occur in the synthesis of a restricted number of proteins during the first 5 hr after exposure of WEHI-3B(D^+) cells to G-CSF. In particular we have examined the changes in nuclear and cytoplasmic proteins by performing cell fractionations using a modification of standard procedures [25]. Protein changes after extended exposure of WEHI-3B(D^+) to G-CSF have been monitored by separating more mature cells using flow cytometry based on cell-surface antigens [35] to allow the identification of differentiation-related cellular proteins.

METHODS

Cells and Culturing

WEHI-3B(D^+) cells were established in this laboratory as a cloned cell line from a mineral-oil-induced myelomonocytic leukemia (WEHI-3) [15]. The cells were continuously subcultured every 7 d in Dulbecco's modified Eagle's (DME) medium, containing 5% v/v heat-inactivated fetal calf serum and were incubated at 37°C with 10% CO_2 in air. WEHI-3B(D^+), which can be induced to differentiate with G-CSF,

should not be confused with the unresponsive subline WEHI-3B(D^-) used to produce hemopoietic regulators [26].

Normal Neutrophils and Macrophages

Neutrophils obtained from the peritoneal cavity of casein-injected mice were purified by the method developed by Watt et al [27]. Macrophages were harvested from the peritoneal cavity of uninjected mice as detailed by Mottram et al [28].

Granulocyte-Colony-Stimulating Factor (G-CSF)

Endotoxin serum (ES) obtained from C57BL mice 3 hr after IV injection of 5 μg bacterial lipopolysaccharide [29] was the starting material for the preparation of granulocyte-colony-stimulating factor. Granulocyte-colony-stimulating factor (G-CSF) was partially purified from ES using gel filtration [as described by 12]. Fifty milliliters of ES purified by gel filtration resulted in 50 ml of G-CSF being obtained from the active fractions. The material was not concentrated before use. This preparation was substantially free of the major species of GM-CSF found in ES, and was used at 2% v/v final concentration in the experiments to be described. At this concentrations, the G-CSF was supramaximal for the formation of differentiated WEHI-3B(D^+) colonies grown as described by Metcalf [11].

Biosynthetic Labeling of Cells

Cells were biosynthetically radiolabled using ^{35}S-methionine (3.7×10^{10} Bq/mmol) (Amersham), at 9.3×10^6 Bq/ml for 3 hr at 37°C in methionine-depleted DME containing fetal calf serum (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. After labeling the cells were washed three times with mouse tonicity phosphate-buffered saline (MTPBS) before preparation for electrophoresis or subcellular fractionation.

Preparation of Nuclei

Cells were placed into an ice-cold hypotonic Tris-HCl buffer (10 mM Tris, pH 7.4, 3 mM $MgCl_2$) for 5 min (10^7 cells/ml) and were lysed using a Dounce glass homogenizer (type B). Seventy-five strokes were sufficient to disrupt most WEHI-3B cells, as gauged by phase-contrast microscopy. The disrupted cells were carefully layered over a sucrose solution (0.65 M sucrose, 10 mM Tris, pH 7.4, 3 mM $MgCl_2$) and centrifugued at 6,000g for 10 min. Cytoplasmic contamination was assessed by measurement of a cytoplasmic enzyme (lactate dehydrogenase) and by phase-contrast microscopy. Yield of nuclei was determined by Hoechst 33258 microfluorimetric assay of DNA [30]. Nuclear pellets were resuspended in the hypotonic solution and recentrifuged through 0.65 M sucrose. This concentration of sucrose were found to prevent intact cells or membranes from pelleting.

Preparation of Cell-Free Extracts for Electrophoretic Analysis

Cells (or nuclei) were suspended in 10 mM Tris-HCl, pH 7.4, at a concentration of 1.5×10^8 cells (or nuclei) per ml. After freezing and thawing three times by immersion into dry ice-ethanol, $MnSO_4$ was added to a final concentration of 0.6 mM to promote double strand cutting of DNA by DNase [31]. Bovine pancreatic DNase I was added to a concentration of 50 μg per ml and the solution was allowed to react at

4°C for 15 min before adding $MgCl_2$ to a final concentration of 5 mM. The cells were solubilized with Triton X-100 (0.5% w/v), and phenyl methyl sulphonyl fluoride (10 mM) and ethylene diamine tetra-acetic acid (20 mM) were added to inhibit proteolysis. The solution was ultracentrifuged at 10^5g in a Beckman Airfuge for 20 min to remove cell debris.

Sample preparation for two-dimensional electrophoretic analysis was by the addition of an equal volume of lysis buffer as described previously by O'Farrell [32]: 1 ml of lysate contained proteins solubilized from 1.5×10^8 cells.

Electrophoretic Analysis

Radiolabeled-proteins from the cells were analyzed using sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (12% acrylamide cross-linked with 0.3% bisacrylamide) by a modification of the Laemmli and Favre procedure [33] utilizing identical cathodal and anodal buffers (25 mM Tris, 0.2 M glycine, 0.1% SDS). The gel dimensions were 75 mm × 68 mm × 1 mm and were poured in glass plates suitable for insertion into a Pharmacia model GE-4 electrophoresis apparatus. The samples were prepared by boiling the cell-free extract for 3 min in an equal volume of sample buffer containing SDS 2.2% w/v, glycerol 15% w/v, and 2-mercaptoethanol 5% v/v. The samples were loaded in a volume of 20 μl (corresponding in each case to 1.5×10^6 cells) and were electrophoresed at 20 mm constant current for 90 min.

The procedures used in this report for the two-dimensional electrophoresis of proteins have been described previously [32], but the apparatus used for the electrophoresis of the proteins was miniaturized to allow greater sensitivity for the detection of proteins by fluorography [34]. The miniature electrophoresis apparatus utilized isoelectricfocusing (IEF) gels poured to a height of 60 mm in 100-μl micropipet tubes (Clay Adams). Ten microliters of protein (derived from 1.5×10^6 cells) was layered on top of the IEF tube with a drawn out fine pipette. The first-dimension IEF gels were electrophoresed at 400 V for 5 hr and were then extruded gently from the tubes by insertion of a 5-μl micropipet tube (Clay Adams) from the acidic end. The fusion between the first and second dimensions involved a further polyacrylamide gel polymerization as it was found that agarose did not provide sufficient mechanical strength to prevent movement of the IEF gel during the second-dimension electrophoresis. The IEF gel was carefully manoeuvered between the glass plates by using a fine spatula, until it was parallel to the second-dimension gel surface, but 5 mm above it. A highly cross-linked acrylamide stacking gel (4.44% acrylamide, 0.8% bisacrylamide) was then poured to cover the IEF gel. Some care was needed to avoid entrapment of air bubbles underneath the gel. O'Farrell's electrophoresis equilibration buffer [32] was modified by not including 2-mercaptoethanol. This allowed polymerization of the stacking gel to occur. The electrophoresis was performed as described for 1D electrophoresis.

Fluorography

Gels were prepared for fluorography by immersion in four times volume of ENHANCE (New England Nuclear, New England) for 45 min with occasional agitation. The scintillant impregnated into the gel was precipitated by transferring to an excess volume of water. The gels were dried under vacuum and exposed for fluorography using Kodak X-Omat RP-5 film. Exposure was at −80°C for 3 d.

Determination of Exposure Densities

A Conalco model J gel scanner was used to determine the exposure density of selected protein spots. Spots were scanned vertically and horizontally and integrated to give a quantitative measure of protein synthesis rates. Spots were scanned from replicate gels and were corrected for any overall differences in exposure density (determined from averaging ten invariant protein spots per gel). The invariant spots were determined by comparison of gels taken from WEHI-3B cells at various stages of differentiation during pilot experiments. In practice, the exposure variation between gels did not usually vary by more than 20% and was therefore easily correctable during the process of numerical integration. All gels were run in triplicate, and any nonreproducible protein variations were not included during the determination of protein synthesis changes during WEHI-3B cell differentiation. Integral spot intensities (corresponding to protein synthesis rates) were expressed in arbitrary units relative to the integral spot intensity in control gels.

Antisera

Rabbit antimouse neutrophil antisera was a generous gift from Dr. S.M. Watt. The antibody was raised in rabbits by the injection of polymorphs purified from mouse peritoneal cells [27] and was mainly of IgM class. It had been consecutively absorbed with equal volumes of packed mouse thymocytes, red blood cells, and unstimulated WEHI-3BM6 cells (60 min on ice). Sheep antirabbit immunoglobulin (a gift from Dr. J. Goding, Walter and Eliza Hall Institute) was conjugated to rhodamine isothiocyanate (RITC) by adding the immunoglobulin (2 mg/ml in MTPBS) in an equal volume of 1 M sodium carbonate buffer (pH 9.5) to 8 μg of RITC (dissolved in dimethylsulfoxide, 2 mg/ml). The reaction was allowed to proceed for 2 hr with gentle agitation at room temperature prior to removing unconjugated dye by passing the mixture through a PD10 column (Pharmacia). The eluate was stored at 4°C in the presence of sodium azide (0.2% w/v). Rhodamine-labeled sheep antirabbit immunoglobulin was utilized as the second antibody. The antiserum was ultracentrifuged at 10^5g in a Beckman Airfuge for 10 min to remove high molecular weight protein complexes prior to staining.

Cell Staining and Sorting

Cells to be sorted were suspended (5×10^6 cells per ml) in 0.3 ml MTPBS-BSA-AZIDE: sodium azide (0.02% w/v), and bovine serum albumin (10 mg/ml) and stained with the absorbed rabbit anti-PMN antiserum (20 μl) on ice for 30 min before centrifugation through 1 ml of FCS and resuspension in 0.3 ml MTPBS-BSA-AZIDE. Use of the buffer solution containing azide was found to prevent adherence of the WEHI-3B(D^+) cells to plastic surfaces and was essential for the maintenance of viability during sorting. [WEHI-3B(D^+) are somewhat unusual in that they die rapidly when incubated at 4°C, unless metabolic inhibitors such as azide are present. The cells must not be allowed to reach room temperature in the presence of azide or this will kill them.] The cells were stained with RITC sheep antirabbit Ig (20 μl) for a further 30 min on ice. A final centrifugation through FCS was performed before resuspension in MTPBS-BSA-AZIDE at 5×10^6 cell per ml.

The cells flowed past a variable argon laser beam of a Becton-Dickinson FACS II cell sorter set to 514 and 488 nm, 0.8 W, with the scatter colinear with the beam, the fluorescence and low-angle scatter being the measured parameters.

RESULTS

Early Initiation of WEHI-3B(D$^+$) Differentiation

WEHI-3B(D$^+$) cells were cultured for 3 hr in the presence of factors inducing differentiation, then transferred for 24 hr to secondary liquid cultures in the presence or absence of inducing factors. The results of three experiments are summarized in Table I. Under all culture conditions there was a threefold increase in cell number after the total culture period. When WEHI-3B(D$^+$) cells were cultured for 3 hr with G-CSF and then transferred to a culture without G-CSF, myelocytes and promonocytes were still produced (26%, Table I). Actinomycin D, which has been shown previously to enhance differentiation induced by G-CSF [35], was no more effective than G-CSF in initiating the production of cells classifiable as promonocytes or myelocytes during the short period of culture (Table I).

The continued presence of either G-CSF or actinomycin D in the secondary culture resulted in a greater degree of WEHI-3B(D$^+$) cell 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 committed within 3 hr. Table I also shows that actinomycin D enhanced the differentiation when cocultured with G-CSF for the entire culture period (68% differentiation).

Actinomycin-D-related cell cytotoxicity was not found during these experiments, although the actinomycin D decreased the rate of WEHI-3B(D$^+$) cell proliferation during the culture period (Table I). The initiation experiments indicated that events important to cellular differentiation were occurring (in a proportion of cells) within 3 hr of treatment. Culture of the short-term (3 hr) G-CSF-treated cells for up to 5 d in the absence of G-CSF indicated that only 5% of the cells had completely matured. The proportion of differentiated progeny gradually decreased during the 5 d of culture in the absence of G-CSF. During this time the cell number increased rapidly, suggesting that the blast cells not committed to differentiation proliferated rapidly, and thus diluted out the cells committed to maturation.

Electrophoretic Analysis of Treated Cells

An examination of the protein changes occurring during the early stages of the commitment period was performed using one- and two-dimensional gel electrophoresis. WEHI-3B(D$^+$) cells were treated with G-CSF for 5 hr and biosynthetically radiolabeled with ^{35}S-methionine during the last 3 hr of treatment. Figure 1 compares fluorographs from untreated WEHI-3B(D$^+$) cells (Fig. 1A) with the G-CSF-treated cells (Fig. 1B). The numbers on the figures indicate the molecular weight of the proteins whose rates of synthesis changed significantly (letters have been used when two proteins with similar molecular weights need to be distinguished). Five proteins were found to be more heavily radiolabeled after G-CSF treatment (proteins 16, 35, 66, 85, and a very large molecular weight protein, labeled vl Fig. 1B). Eight proteins were found to be radiolabeled to a decreased extent (29, 32, 33, 34, 35a, 36, 67, and 84). The decreases in radiolabel incorporation were found predominantly in acidic proteins (ie, pI 4.8): 29, 32, 33, 34, 35a, 36, 67, and 87 have isoelectric points close to 4.4. A protein of 16k daltons (number 16 in Fig. 1B) exhibited a large increase in label incorporation (6.4-fold greater than in the untreated control cells; Fig. 1A). The increases and decreases of the label incorporated into some proteins compared to control cells are summarized in Table II.

TABLE I. Transfer of WEHI-3B (D^+) Cells From Primary to Secondary Cultures: Initiation of Differentiation

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-hr duration: There were 4.9×10^6 in 20 ml of culture medium before stimulation.
[b]Secondary culture was of 24 hr 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.
[e]Actinomycin D was used at 5 ng/ml final concentration.

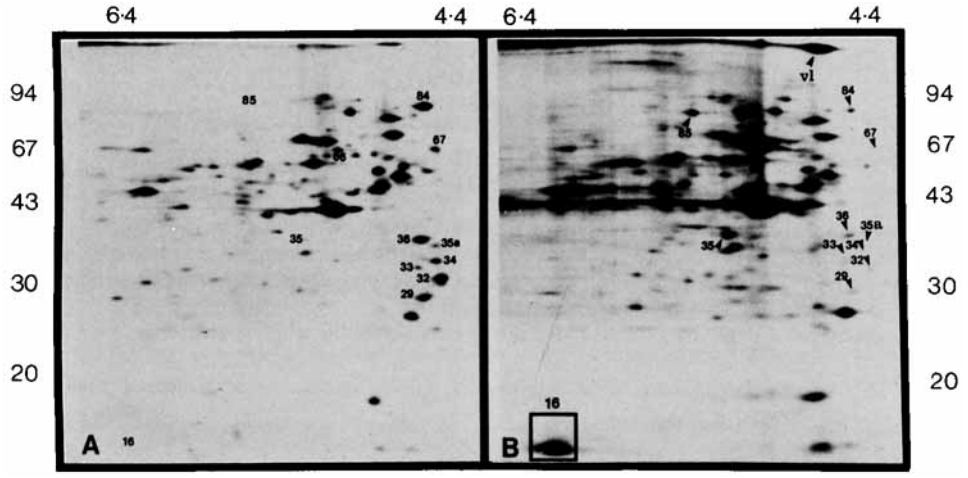

Fig. 1. WEHI-3B(D^+) cells were treated for 5 hr with A) normal saline (NS) 2% v/v or B) G-CSF 2% v/v and were radiolabeled with ^{35}S-methionine during the last 3 hr of treatment before 2D gel electrophoresis and fluorography. Arrows indicate increases or decreases of protein radiolabel uptake, induced by G-CSF relative to the NS treated controls. Numerals associated with the arrows indicate molecular weight of proteins.

It might be argued that the decreased ^{35}S-methionine radiolabeling observed for some proteins reflects an increased level of protein degradation rather than decreased protein synthesis. This was tested by a pulse-chase experiment: WEHI-3B(D^+) cells were labeled with ^{35}S-methionine in the presence or absence of G-CSF for 3 hr, then recultured in normal medium without radioisotope or G-CSF for a further 3, 5, and 19 hr. As the acidic proteins between 25k and 38k daltons had been found to be particularly sensitive to G-CSF treatment (Fig. 1), the regions of fluorographs con-

TABLE II. Changes of ^{35}S-Methionine Incorporation Into WEHI-3B (D$^+$) Cellular Proteins During G-CSF Treatment*

Protein MW (kilodalton)	Source	Analytical technique	Radiolabel incorporation relative to untreated cells[a]
120 (vl)	Whole cell	IEF 2D	3.20 + 1.2
85	Whole cell	IEF 2D	4.10 + 0.7
85	Whole cell NHP	1D	3.40 + 0.4
84	Whole cell	IEF 2D	0.26 ± 0.03
67	Whole cell	IEF 2D	0.21 ± 0.02
66	Whole cell	IEF 2D	2.10 ± 0.4
36	Whole cell	IEF 2D	0.31 ± 0.04
36	Nucleus	1D	0.32 ± 0.05
36	Nucleus	IEF 2D	0.18 ± 0.04
35a[b]	Whole cell	IEF 2D	0.43 ± 0.03
35	Whole cell	IEF 2D	2.50 ± 0.3
34	Whole cell	IEF 2D	0.24 ± 0.04
32	Whole cell	IEF 2D	0.29 ± 0.04
29	Whole cell	IEF 2D	0.27 ± 0.03
24	Nucleus	IEF 2D	0.34 ± 0.04
20	Whole cell	IEF 2D	1.60 ± 0.3
20	Whole cell NHP	1D	2.10 ± 0.3
20	Nucleus	1D	0.35 ± 0.05
18	Whole cell	IEF 2D	1.70 ± 0.03
18	Nucleus	IEF 2D	0.38 ± 0.03
17	Nucleus	1D	0.35 ± 0.06
17	Nucleus	NEPHGE 2D	0.27 ± 0.03
16a[b]	Whole cell	IEF 2D	6.40 ± 0.06
16	Nucleus	1D	0.75 ± 0.06
16	Whole cell NHP	1D	2.20 ± 0.3

*Intensity of spots determined by scaning spot (with Conalco gel scanner) in two directions, then integrating. Figures were corrected for slight background variations determined from scanning several invariant spots. Figures are means from three gels and are expressed as a ratio relative to uninduced WEHI-3B(D$^+$) proteins, plus or minus the standard deviation.

[a]Cells were treated with G-CSF (2% v/v) for 5 hr before radiolabeling with ^{35}S-methionine for a further 3 hr.

[b]Suffix "a" identifies the protein as acidic to distinguish it from proteins of equal molecular weight.

taining these protein spots were excised and photographed to allow direct comparison (Fig. 2). Untreated WEHI-3B(D$^+$) cells synthesized many acidic proteins (Figs. 1A, 2A). WEHI-3B(D$^+$) cells cultured with ^{35}S-methionine for 3 hr, then cultured for a further 3 hr in normal medium, were found to have four medium molecular weight acidic proteins (29, 32, 35a, and 36) radiolabeled to a decreased extent, implying that rapid degradation (ie, normal turnover) of these proteins was occurring (Fig. 2B). Acidic proteins from radiolabeled cells cultured for a further 5 and 19 hr in normal medium before electrophoresis analysis are shown in Figure 2C and D, respectively. Further protein turnover was evident: Proteins 29 and 32 had lost most of their label by 5 hr (Fig. 2C) and by 19 hr proteins 34 and 26 were also significantly degraded (Fig. 2D). Similar pulse-chase experiments utilizing G-CSF-treated WEHI-3B(D$^+$) cells revealed that the initial decreases in radiolabel incorporation (Fig. 1B) were a combination of inhibition of protein synthesis and of specific protein degradation. Proteins 29, 33, and 36 were synthesized to a significantly decreased extent during 3

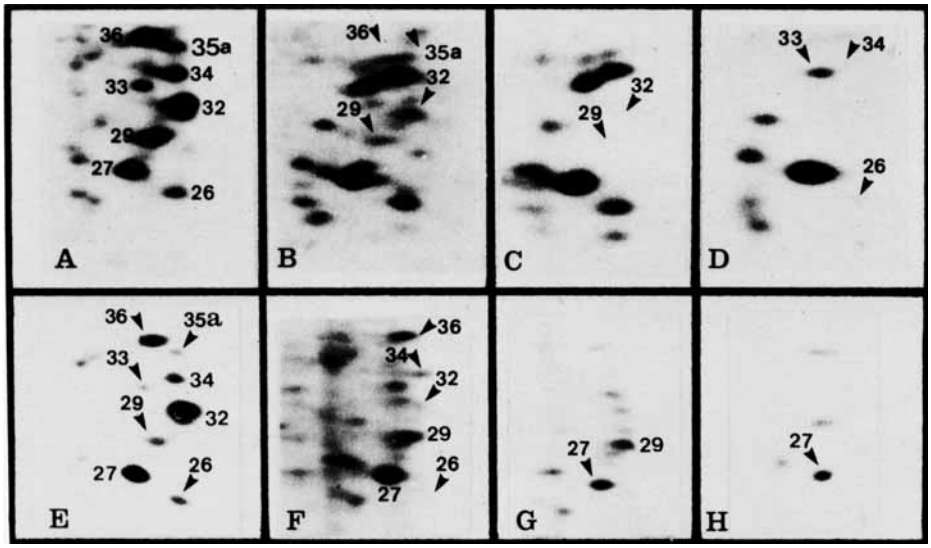

Fig. 2. Fluorographs of acidic proteins between 25k and 40k daltons from untreated (A–D) or G-CSF-treated (E–H) cells simultaneously radiolabeled for 3 hr with ^{35}S-methionine. Cells were cultured in medium for a further A) 0 hr; B) 3 hr; C) 5 hr; D) 19 hr; E) 0 hr; F) 3 hr; G) 5 hr; and H) 19 hr after radiolabeling, then prepared for 2D electrophoresis and fluorography.

hr of G-CSF treatment (Fig. 2E vs 2A), but proteins 33 and 36 did not exhibit different rates of protein degradation (as determined from the relative decrease in ^{35}S-methionine during the "chase" period (Fig. 2E–H vs 2A–D)). Protein 29, although synthesized to a decreased extent after G-CSF treatment, appeared to exhibit a decreased turnover rate compared to the untreated cells (Fig. 2E–G vs 2A–C). Proteins 26, 27, 32, and 34 were degraded more rapidly during G-CSF treatment as determined by the reduction in radioisotope during culture in normal medium after prior G-CSF treatment. Comparison of Figures 1B, 2E, and 2F revealed that the synthesis of protein 32 (rapidly degraded after labeling in both G-CSF treated and untreated cells) was suppressed between 3 and 5 hr after initiation of G-CSF treatment (compare Figs. 1B and 2E).

Thus the synthesis of four proteins (29, 32, 33, and 36) was specifically suppressed by G-CSF, whereas the rate of degradation of four proteins (26, 27, 32, and 34) was increased. This combination of decreased protein synthesis and increased protein degradation induced by treatment of WEHI-3B(D$^+$) cells with G-CSF for 3 hr accounted for all the decreased ^{35}S-methionine incorporations observed in Figure 1B (2 hr of G-CSF treatment followed by 3 hr of radiolabeling in the presence of G-CSF).

Protein Profiles of Blast and Mature WEHI-3B(D$^+$) Cells

Purified mature WEHI-3B(D$^+$) macrophages were obtained after FACS sorting from a heterogeneous maturing population of cells derived from treating WEHI-3B(D$^+$) cells for 6 d with G-CSF [35]. These cells stained positively for α-napthylacetate esterase, were phagocytic, loosely adherent (ie, easily removed by pipetting), morphologically mature (Fig. 3), and 90% pure. These macrophages were separated

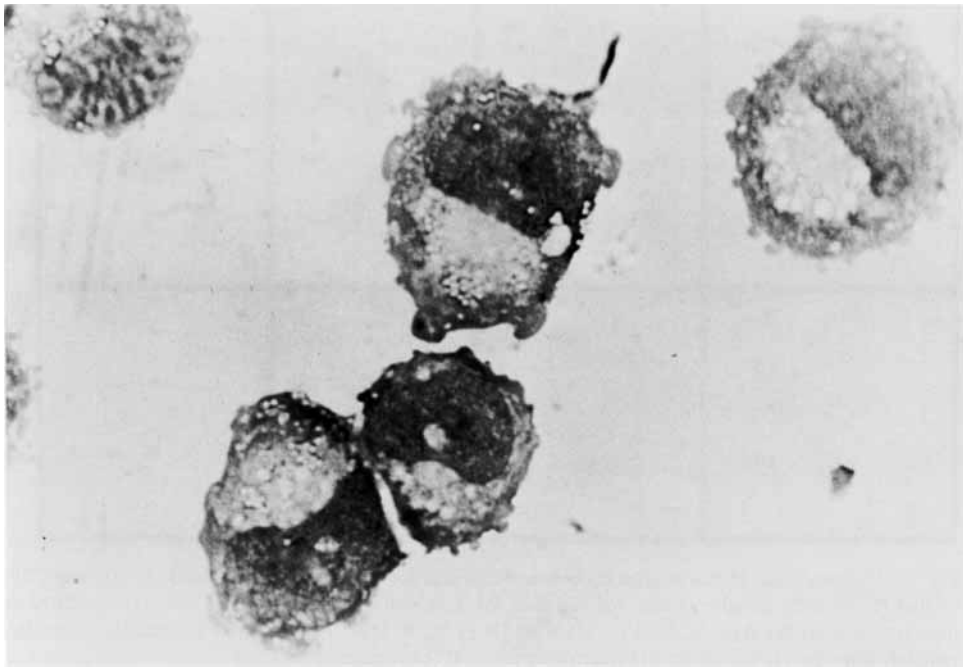

Fig. 3. Phagocytic macrophages derived by fluorescence-activated cell sorting of 6-d G-CSF-treated WEHI-3B(D^+) cells were cultured with latex beads for 3 hr. ×1,000.

from immature WEHI-3B(D^+) cells by sorting on the basis of high fluorescence (channels 170–300) with a rabbit antipolymorph and monocyte antibody. Electrophoretic analysis revealed many protein differences between these macrophages (Fig. 4B) and WEHI-3B(D^+) blast cells which had been G-CSF treated for 5 hr (Fig. 4B and A, respectively). Eight proteins appeared to be synthesized de novo (eg, 30 and 31) in the macrophages whilst a further seven were increased compared to the G-CSF-stimulated WEHI-3B(D^+) blast cells. In particular, proteins 16 and vl were increased 3.2-fold and 2.3-fold, respectively, in the WEHI-3B(D^+) macrophages compared to the G-CSF-treated blast cells. These proteins are therefore examples of late differentiation markers. The FACS-purified macrophages were radiolabeled in the same set of experiments as detailed in Figure 1A and B; hence the gel from the G-CSF-stimulated cells (Fig. 1B) was used for comparison in Figure 4A. A protein of 18k daltons (18—Fig. 4B) was the only protein to show a decreased label content compared to the treated blast cells. It is therefore apparent that many proteins in the leukemic blast cells were still actively synthesized in the mature cells, but also that several proteins with increased isotopic uptake during early differentiation (compare Fig. 1A and B) were found to be highly radiolabeled in the mature macrophage (eg, proteins 16a and vl). Protein 35, whilst showing increased labeling during initial G-CSF treatment (Fig. 1B), did not show any further increase in biosynthetic rate in the WEHI-3B(D^+)-derived macrophages. In contrast, however, WEHI-3B(D^+)-derived myelocytes (purified to 91% by selecting channels 50–110 on the FACS from G-CSF-treated cells) synthesized protein 35 at almost ten times the rate of WEHI-3B(D^+)

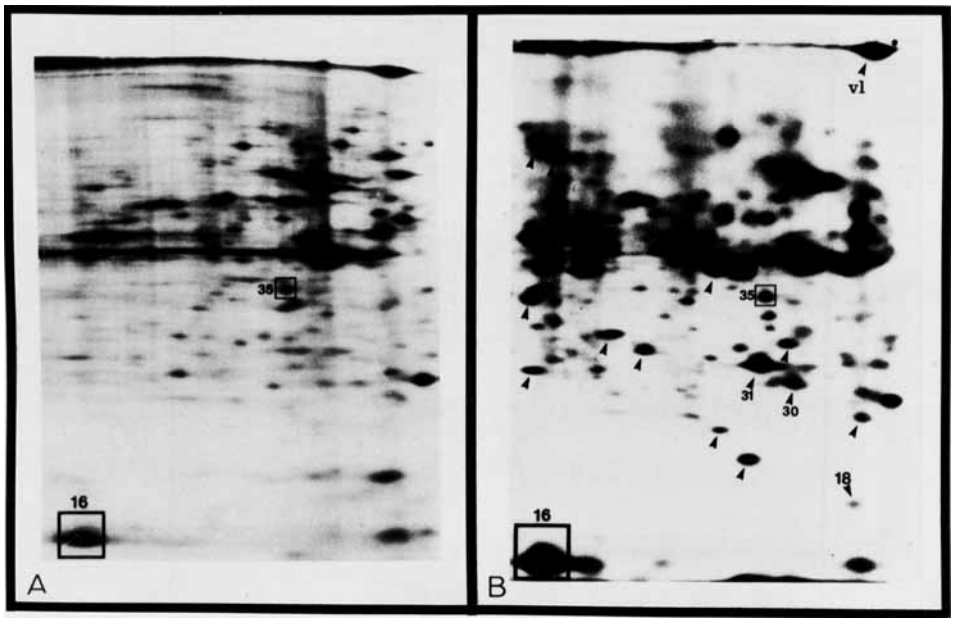

Fig. 4. A) WEHI-3B(D^+) cells treated with 2% v/v G-CSF for 5 hr were radiolabeled for the last 3 hr of treatment with ^{35}S-methionine (see also Fig. 1B). B) WEHI-3B(D^+) cells treated with 2% v/v G-CSF for 6 d were purified by fluorescence-activated cell sorting and were classified as 90% pure macrophages. These cells were radiolabeled for 3 hr with ^{35}S-methionine.

blast cells or macrophages (Fig. 5A). Two induced proteins were common to both the purified myelocytes and macrophages: protein 31 and 30 (compare Fig. 5B and A). Protein 35 appeared to be more actively synthesized in myelocytes than macrophages (Fig. 5B vs A).

Analysis of Nuclear Proteins

The nuclei from ^{35}S-labeled WEHI-3B(D^+) cells 3 hr and 5 hr after G-CSF-initiated cell differentiation were purified and the radiolabeled proteins analyzed by electrophoresis. The nuclear pellet separated by sucrose density centrifugation from intact WEHI-3B(D^+) cells and membranes was tested for the presence of lactate dehydrogenase. This enzyme has been previously used as a cytoplasmic enzyme marker and thus acts as an indicator of nuclear purity [36]. After two centrifugation steps, the level of contamination of nuclei with cytoplasm was less than 1% (Table III), whilst the recovery of nuclei was greater than 90% as determined from total DNA measurements using Hoechst 33258 dye binding (data not shown).

The densitomer tracing of the 1D nuclear protein fluorographs from 12% polyacrylamide gel electrophoresis (Fig. 6) indicated a pattern of radiolabeling changes consistent with some of the changes found in the whole cell extracts of treated WEHI-3B(D^+) cells (Fig. 1, Table II). For example, a set of proteins in the region of 36k daltons was found to be suppressed or degraded in the whole cell extracts (Figs. 1, 2) after G-CSF treatment, and a set of similar molecular weight nuclear proteins rapidly decreased their incorporation of radiolabel as shown by the densitometric profiles

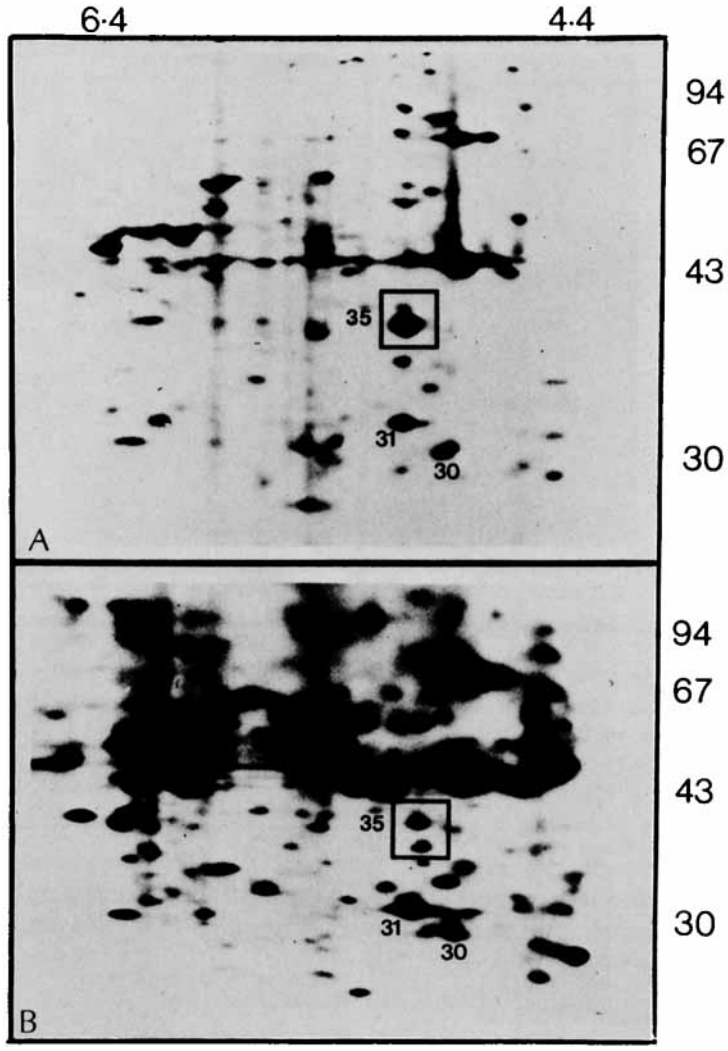

Fig. 5. A) WEHI-3B(D^+) myelocytes (91% pure) were obtained from WEHI-3B(D^+) cells induced to differentiate by treatment for 6 d with G-CSF, and were purified by fluorescence-activated cell sorting. The myelocytes were biosynthetically radiolabeled with ^{35}S-methionine for 3 hr before analysis by 2D electrophoresis and subsequent fluorography. B) WEHI-3B(D^+) cells treated with 2% v/v/ G-CSF for 6 d were purified by cell sorting and were 90% pure macrophages (see also Fig. 4B).

(Fig. 6A–C). There were many similarities between the 1D nuclear protein profiles and the whole cell extracts, with the most prominent being the synthesis of actin in both samples (protein 43 in Fig. 6). However, many of the protein synthesis increases observed in the cell extracts could not be related to nuclear protein changes. For example, a protein of 16k daltons was extensively synthesized during G-CSF treatment (Fig. 1B; increasing 6.4-fold), but the nuclear proteins of equivalent molecular weight showed a clear 1.3-fold decrease in radiolabel incorporation (Fig. 6, Table II). Similarly, two decreases in protein synthesis observed by 1D electrophoresis of

TABLE III. Lactate Dehydrogenase Activities in Nuclear and Cytoplasmic Fractions of WEHI-3B (D^+) Cells

Experiment No.	Fraction	Specific activity (units per mg)[a]	Total activity in fraction	Percent cytoplasmic contamination[b]
1	Cytoplasm	24.5	2,450	
1	Nucleus[c]	0.75	3	0.1
2	Cytoplasm	17.5	1,750	
2	Nucleus	3.5	14	0.8
3	Cytoplasm	6.5	650	
3	Nucleus	0.8	3	0.5

[a]One unit of activity is that which results in the oxidation of 1 μmol of $NADH_2$ per min at 25°C.
[b]Calculated as total nuclear activity divided by total cytoplasmic activity × 100.
[c]Recovery of nuclei was measured by staining with Hoechst 33258 and found to be 90%.

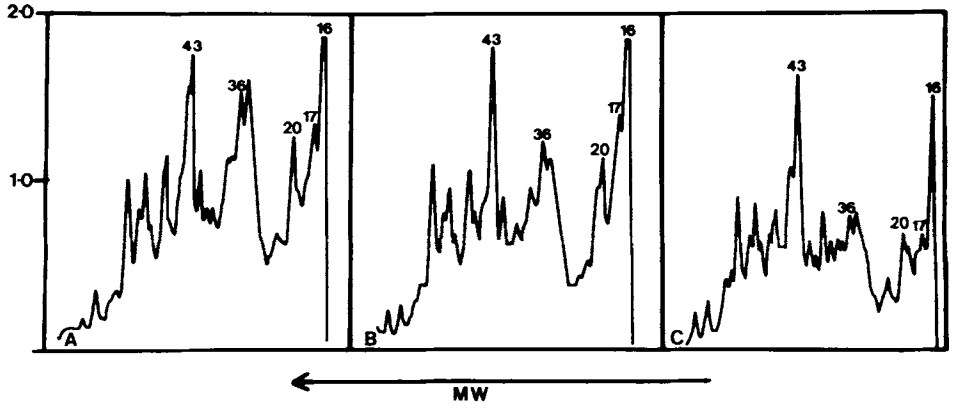

Fig. 6. Nuclei were purified from WEHI-3B(D^+) cells which had been treated with G-CSF (2% v/v) for A) 0 hr; B) 3 hr; and C) 5 hr, then radiolabeled for 3 hr with ^{35}S-methionine. The proteins were electrophoresed on 1D SDS-gels and fluorographed. The fluorographs were scanned from high to low molecular weight (left to right) using a Conalco gel scanner. Numbers above peaks indicate molecular weight and the ordinate is marked in arbitrary absorbance units.

nuclear proteins (Nos. 17 and 20 decreasing 2.9-fold relative to controls—see Table II) could not readily be associated with whole cell protein changes (Fig. 1). Such proteins might therefore be localized to the cytoplasm, or alternatively they might be too basic to be resolved by IEF electrophoresis.

Two experimental strategies were developed to examine these possibilities. First, the basic proteins in whole cell lysates were examined by 1D electrophoresis, and second, proteins synthesized in the nuclear compartment were examined using techniques of higher resolution.

Analysis of Nonhistone Cellular Proteins

Nuclear proteins comprise both basic proteins (eg, histones) and acidic proteins, and it was considered possible that extensive synthesis decreases in the basic proteins

might mask any nonhistone (acidic) protein biosynthesis, due to a failure to resolve them by 1D electrophoresis.

Nonhistone proteins (NHP) were therefore extracted from whole cell lysates and analyzed by 1D gel electrophoresis. The NHPs were separated from histones by acid extraction [37] which removed the most basic proteins (predominantly histones), and the remaining proteins were then analyzed by 1D electrophoresis. Figure 7 shows the electrophoretic profiles of NHPs separated from WEHI-3B(D^+) cells stimulated with G-CSF for 5 hr (Fig. 7B) compared to untreated WEHI-3B(D^+) NHPs (Fig. 7A). These results indicated clearly that in contrast to the 1D electrophoretic analysis of the total nuclear protein profiles (Fig. 6), there was an increased biosynthesis of a protein of 16k daltons (increasing 2.2-fold), a protein of 75K daltons (increasing 3.4-fold), and a protein of 20k daltons (increasing 2.1-fold; Fig. 7B). These protein synthesis changes appear closely related to the increased labeling of proteins 16 and 20 (thus the 20 dalton protein has an isoelectric point similar to the very large protein (marked vl in Fig. 1) detected by 2D electrophoresis of total cellular components (Fig. 1, Table II). The protein of 75k daltons could not be localized to any one spot in the 2D IEF gels of the whole cell proteins (Fig. 1).

2D Gel Analysis of Acidic Nuclear Proteins

The results obtained from the 1D nuclear protein densitometric profiles (Fig. 6, 7) strongly suggested that a decreased synthesis (or increased degradation) of proteins 16 and 17 must occur in a subset of proteins not detected by the resolution of NHPs. The nuclear proteins were therefore examined using techniques with higher resolution, examining both acidic and basic proteins.

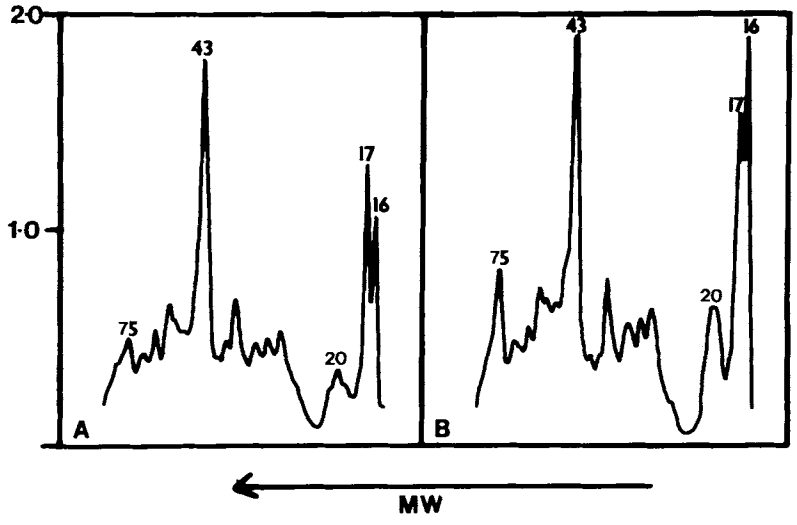

Fig. 7. Nonhistone proteins (NHP) extracted from ^{35}S-methionine-radiolabeled cells (3 hr) previously treated with G-CSF (2%) for A) 0 hr and B) 5 hr were electrophoresed on 1D SDS-gels and then fluorographed. The fluorographs were scanned using a Concalco gel scanner. Numerals above the peaks indicate molecular weight and the ordinate is marked in arbitrary absorbance units.

The nuclear proteins of WEHI-3B (D^+) cells were subjected to 2D gel electrophoresis in an attempt to further define the important early events in cellular differentiation. The radiolabeling pattern of the nuclear proteins was apparently simpler than that for the whole cell lysates (cf, Fig. 8 and 1). Actin was found to be present amongst the nuclear proteins (Fig. 8A, protein labeled "act") as has also been reported for rat liver cells [38]. The resolution of proteins at the basic end of the gel was poor in contrast to the whole cell extracts, but the reason for this is not clear at present. A most striking feature of the radiolabeling patterns was the rapid decrease of a protein of 36k daltons to 0.18 of original level (Fig. 8B) during 5 hr of differentiation induced by G-CSF. The radioisotope content of an acidic protein of 18k daltons also decreased to 0.38 of original level during this regime (Fig. 8B), in agreement with the 1D nuclear protein data—see Table II. A protein of 24k daltons (Fig. 8B) was not found to be a major protein in the whole cell lysates (Fig. 1) but was suppressed in the nucleus during G-CSF treatment to 0.34 of its original synthesis rate (Table II).

Of particular interest, however, was the relative paucity of nuclear protein changes: of 100 proteins detectable, only four (18, 24, 36, and 53) had significantly decreased incorporation of radioisotope and only one increased its incorporation (57, Fig. 8B) during G-CSF treatment, in contrast to the many changes detectable in whole cell lysates (Fig. 1). The 36k dalton protein synthesis decrease (Fig. 8B) was particularly large and indicated that the same change observed in the whole cell lysates (Fig. 1) may have been due to the decreased labeling of this nuclear protein. Gel scans of cytoplasmic proteins after removal of nuclei revealed only trace quantities of this protein (data not shown), supporting this hypothesis.

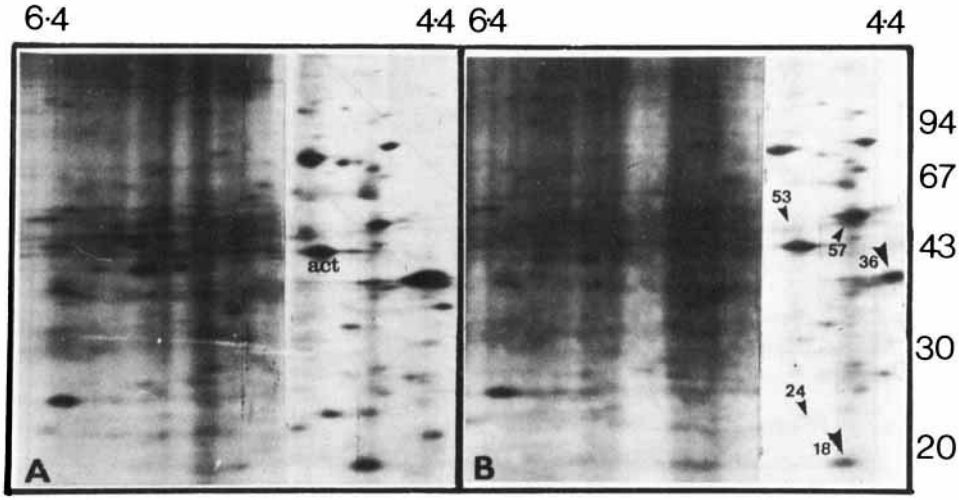

Fig. 8. Nuclei were purified from WEHI-3B(D^+) cells which had been radiolabeled with ^{35}S-methionine for 3 hr after treatment with A) NS (2%) for 5 hr and B) G-CSF (2%) for 5 hr. Molecular weights are marked in kilodaltons on the ordinate; pH units are marked on the abscissa. Actin is marked by the symbol "act" in (A).

2D Analysis of Basic Nuclear Proteins

Nonequilibrium pH gradient electrophoresis (NEPHGE) developed by O'Farrell [39] was used to further analyse the protein synthesis changes occurring in the low molecular weight nuclear proteins of G-CSF-treated WEHI-3B(D^+) cells. Figure 9 shows the low molecular weight proteins selected from fluorographs of nuclei resolved by NEPHGE and indicates the region being examined during G-CSF treatment. The pH units are approximate only, being determined by pH measurement run on comparable large gels. G-CSF treatment resulted in a rapid decrease in labeling of basic 17k and 16k dalton proteins occurring between 3 hr and 5 hr after stimulation (decreasing to 0.27 of control rate for protein 17), suggesting that the decreased incorporation of label observed in 1D analysis of nuclear proteins of equivalent molecular weight was due to the decreased synthesis of this basic proteins (cf Fig. 6).

Protein 20, which was found by 1D electrophoresis to have a decreased content of radiolabel during G-CSF treatment of cells (Fig. 6) was not localized to a particular spot on the NEPHGE gels. It is possible that protein 20 (resolved by 1D electrophoresis) may actually comprise more than one protein of widely different isoelectric points. The failure to detect such heterogeneous proteins by 2D gel electrophoresis (IEF or NEPHGE) might suggest that one protein component was too basic to be resolved even by NEPHGE techniques. The decreased incorporation of isotope into the basic protein 16 (Fig. 9) was not sufficient to account for the decreased radiolabeling detected by 1D electrophoresis of the nuclear proteins, which suggests that the same possibility may exist for protein 16a as for protein 20.

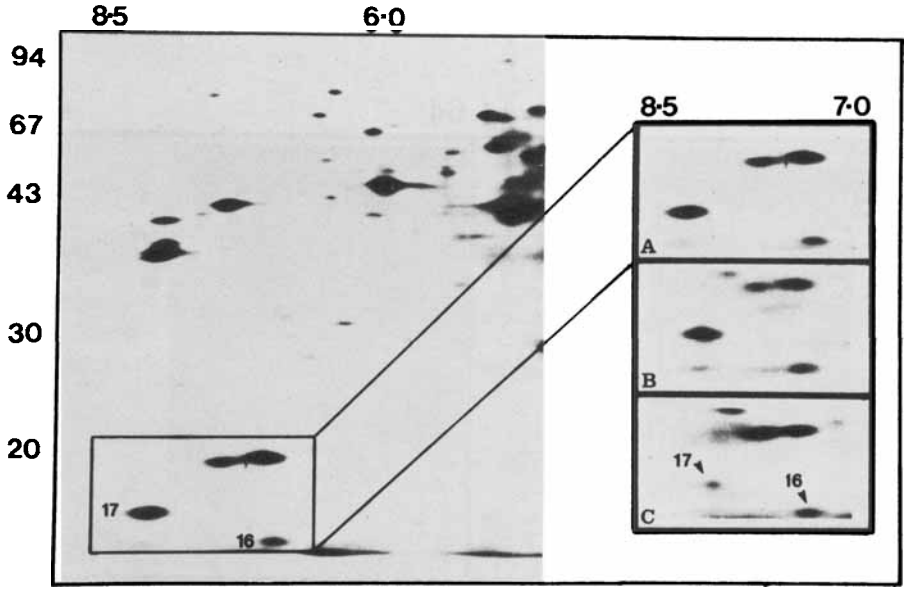

Fig. 9. Nuclear proteins from radiolabeled WEHI-3B(D^+) cells were resolved by 2D-NEPHGE techniques. Regions of fluorographs corresponding to low molecular weight basic proteins are depicted and a complete NEPHGE gel shows the regions being compared. The proteins were derived from cells treated with A) G-CSF (2%) for 0 hr; B) G-CSF (2%) for 3 hr; and C) G-CSF (2%) for 5 hr, and radiolabeled for 3 hr with ^{35}S-methionine. Approximate pH units are marked on the abscissa.

Intracellular Localization of Differentiation-Related Proteins

Comparison of NEPHGE, IEF, and 1D gel fluorographs enabled some of the major proteins involved in early synthesis changes to be localized to the cytoplasm or nucleus. Figure 10 summarizes schematically some of the data tabulated in Table II and indicates the localization of the protein and approximate magnitude of its alteration after 5 hr of treatment with G-CSF. Protein 85, for example (85k daltons), was not detected in the nucleus, but was found in whole cell lysates; hence it was deduced to be a cytoplasmic protein. Similarly proteins 36 and 35 were assigned to the nucleus on the basis that the nuclear gels revealed very large synthesis changes in these proteins when compared to whole cell lysates. The presence of these proteins in the cytoplasm was not positively excluded, however, and they are therefore depicted on the nucleus/cytoplast boundary (Fig. 10).

Protein 16 was not detected in nuclear IEF gels but an equivalent molecular weight protein was present in nuclear proteins resolved by 1D electrophoresis (Fig. 6, Table II). Protein 16 was found to be rapidly synthesized in whole cell lysates of G-CSF-treated cells run on IEF gels, but less rapidly in 1D gels of NHP (Fig. 7, Table II). Taken together, these data suggest that protein 16 resolved by 1D gel electrophoresis is in fact two proteins, one acidic and the other a basic nonhistone protein. Furthermore, the large radiolabeling change in the acidic protein (16) de-

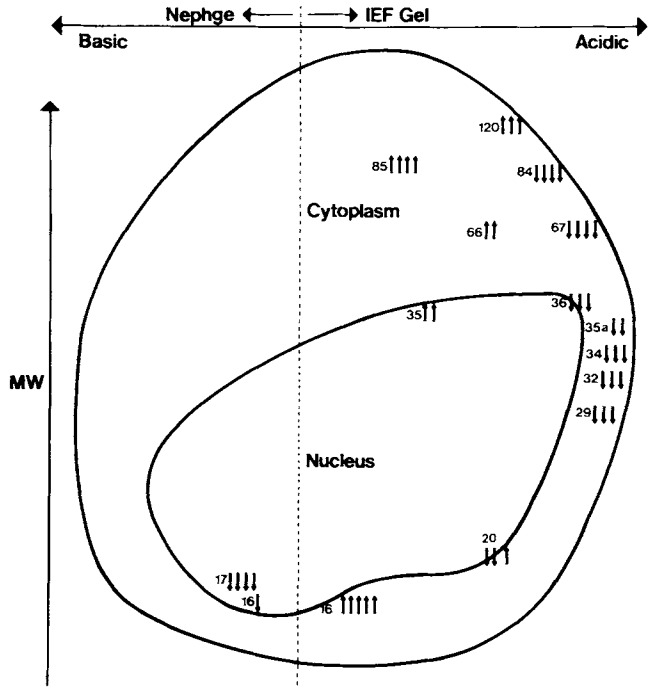

Fig. 10. The protein changes quantified in Table II are presented in a schematic form indicating the magnitude of the change of ^{35}S-methionine incorporation, the molecular weight, the acid/base nature of the protein, and its location in the nucleus and/or the cytoplasm. Proteins depicted on the nucleus/cytoplasm boundary could not be localized to either compartment.

tected in whole cell lysates by IEF (Fig. 1) but not in nuclear proteins (Fig. 8) suggests that one of the two proteins is a cytoplasmic protein (Fig. 10).

A similar hypothesis is also proposed for proteins 20 and 17. To date, the basic protein postulated to be of 20k daltons has not been resolved, but proteins 16 and 17 were detected on NEPHGE gels which resolved nuclear proteins, and were thereby identified as G-CSF-suppressed basic nuclear proteins (Fig. 9).

DISCUSSION

The results of the culture transfer experiments indicated that up to 29% of WEHI-3B(D^+) cells could be initiated to differentiate by 3 hr of treatment with G-CSF. The data also indicated that a longer treatment with G-CSF or actinomycin D and G-CSF (up to 24 hr) induced more cells to form myelocytes and promonocytes, indicating that not all cells capable of responding to G-CSF were able to do so within 3 HR of treatment. After short-term incubation of WEHI-3B(D^+) cells with G-CSF (3 hr), further culture of the cells for 5 d in the absence of G-CSF resulted in only a small proportion of mature cells (5%). This could be interpreted to mean that the initial differentiation induced by a 3-h exposure to G-CSF (assessed at 1 d) is partly reversible after removal of regulator (as postulated for dimethylformamide-induced differentiation of HL-60 cells [40]. However, it is also possible that blast cells not committed to differentiation were able to proliferate faster than the maturing cells, thus resulting in an apparent decrease in the percentage of mature cells. Some studies have indicated that leukemic cell lines may be reversibly differentiated; eg, K-562 erythroleukemia synthesizes hemoglobin (a differentiation marker) only in the continued presence of hemin, and such synthesis is rapidly and reversibly suppressed in its absence [41]. Other experiments utilizing hemoglobin production as a measure of cell maturity in Friend cells have indicated that irreversible differentiation occurs only during the G1 phase of the cell cycle and that cells had to proceed through that phase before commitment to hemoglobin-producing cells occurred [42]. It is not clear from our experiments whether reversible differentiation occurred for WEHI-3B(D^+) cells, but the gradual decrease in the percentage of mature cells during culture (data not shown) tends to suggest that the mature cells were merely growing at a slower rate after G-CSF treatment, thus being gradually supplanted by the faster-growing blast cells. Recent experiments using HL-60 cells showed that the initiation of cellular differentiation induced by dimethylsulfoxide (DMSO) was independent of the cell cycle, was irreversible, was proportional to the length of time the cells were exposed to the inducing agent, and did not require cell division for manifestation of the differentiated cell phenotype [48]. The authors determined that the cells required contact with inducer for between 6 and 12 hr for manifestation of differentiation (detected by morphology and functional activity). Based on their data, the doubling time for the HL-60 cells was 38 hr, implying that the cells required contact with DMSO for between 0.16 and 0.32 of their cell cycle period before the initiation of any cell differentiation [48]. WEHI-3B(D^+) cells exhibited a doubling time of 14 hr; the equivalent fractions of cycle times necessary for the initiation of differentiation (based on the HL-60 cell data) would be between 2.2 and 4.4 hr. As we observed commitment of some of the WEHI-3B(D^+) cells after 3 hr of treatment with G-CSF, the data appear to be consistent with that reported for the HL-60 cells. Limit dilution cultures might clarify this possibility by preventing the overrunning of cultures by

immature cells (eg, blasts), thus allowing the morphology of the cells to be accurately assessed in response to short stimulations with G-CSF. Alternatively, the sorting of maturing cells (on the basis of fluorescence) 24 hr after treatment with G-CSF, followed by growth in agar, might be expected to determine how rapidly irreversible commitment to differentiation occurs. Staining of DNA would enable sorting of cells in different stages of the cell cycle [42], thus providing information on whether commitment is restricted to a particular phase of the cycle.

The experiments reported in this manuscript have analyzed the whole cell and nuclear protein changes found to occur in a murine myelomonocytic leukemia cell line (WEHI-3B(D^+)) during induction of differentiation with G-CSF. [Although most of this work has used unfractionated G-CSF preparations, it should be noted that identical changes were induced with preparations of G-CSF that had been purified 100-fold.] The program of differentiation with these inducing agents gave rise to macrophages which have been previously shown to express mature cell antigens, to phagocytose latex beads, and to synthesize lysozyme [35]. The protein changes occurring within the first few hr of the induction of differentiation were examined to determine if a particular protein or group of proteins was involved in the initial cellular commitment process. The data from pulse-chase experiments showed that the protein turnover was rapid for many proteins in WEHI-3B(D^+) cells, and suggested that certain proteins (in particular the acid proteins 26, 27, 32, and 34) may have been specifically degraded during the early processes occurring in the cellular differentiation (Fig. 1, 2), whereas the rate of synthesis of proteins 29, 32, 33, and 36 was suppressed.

Further examination of whole cell lysates from radiolabeled WEHI-3B(D^+) cells showed that six proteins increased their synthesis rates during brief treatment with G-CSF (proteins vl, 85, 66, 35, 18, and 16). Comparison with fluorescence-purified WEHI-3B(D^+)-derived macrophages revealed that many proteins were synthesized to a greater extent in the macrophages and that several proteins appeared to be synthesized de novo. Furthermore, the changes in synthesis found in the blast cell proteins during brief G-CSF treatment were, for the most part, further enhanced in the macrophage; eg, proteins vl, 85, 66, and 16a had further increased rates of synthesis, while proteins 84, 67, 36, 35a, 34, 32, and 29, which had decreased synthesis in the blast cells during G-CSF treatment, also decreased further in the macrophages. Protein 35, however, which increased synthesis in the blast cells, did not undergo further change in the macrophages. Furthermore, protein 35 was found to be a major constituent of WEHI-3B(D^+)-derived FACS-purified myelocytes (Fig. 5). Thus this protein may be associated with the early differentiation events. In contrast proteins vl and 16 appeared to be mature cell markers and have been found in normal neutrophils [43] and maturing M1 cells [23].

Analysis of purified nuclear proteins by 1D and 2D electrophoresis revealed some protein synthesis changes detectable by 1D analysis, but not on the usual IEF 2D gels. To further clarify these data, NEPHGE gels able to resolve basic proteins [39] were utilized and 1D analysis of NHPs was performed. The data indicated that certain protein changes found to occur by 1D electrophoresis corresponded to basic proteins (eg, protein 16 and 17), and furthermore that several of the protein changes apparent in the whole cell extracts were not found in the nucleus (eg, proteins 85, 84, 67, and 66). It was also found that some protein synthesis changes occurring in whole cell lysates were probably attributable to nuclear events (eg, proteins 85, 84, 67, and

66). These data are summarized in Figure 10. The data also revealed that while the number of protein changes occurring in the nucleus appeared small by 2D IEF electrophoresis (relative to the whole cell lysates), the magnitude of the changes observed suggested that the nuclear events could not be ignored for a thorough analysis of cellular differentiation. The apparently conflicting results obtained by 1D analysis of NHP and nuclear proteins (eg, protein 17) was resolved by careful comparison with IEF and NEPGHE 2D techniques and revealed the importance of analyzing basic proteins: Protein 17 was resolved by NEPHGE and showed a synthesis decrease during G-CSF treatment of WEHI-3B(D^+) cells (Fig. 9). The conflicting data obtained also for protein 18 by 1D and 2D analysis of proteins (Table II) was partly resolved by the analysis of the nuclear proteins: Protein 18 appears to be present in both nuclei and cytoplasm but is synthesized more rapidly in the cytoplasm during G-CSF treatment while disappearing from the nucleus. Either this may represent a combined cytoplasmic protein synthesis and migration from the nucleus to the cytoplasm or the two events may be unrelated. Further analysis of cytoplasmic proteins will be necessary to determine which hypothesis is correct.

Our data indicate, however, that relatively few protein changes were observed for WEHI-3B(D^+) cells undergoing differentiation. The protein changes summarized in Table II and Figure 10 are far fewer than those reported to alter in M1 cells undergoing differentiation [16]: Whereas 14 G-CSF-induced protein synthesis changes were detectable by IEF 2D electrophoresis, and a further four by examination on NHP, basic, and nuclear proteins in WEHI-3B(D^+) cells, 50 proteins changed in M1 cells induced by MGI (containing GM-CSF and G-CSF) resolved by normal IEF 2D electrophoresis after similar periods of stimulation (3–5 hr). It is perhaps relevant to note that other studies have reported relatively few protein changes during induced cellular differentiation, eg, erythroleukemia cells treated with DMSO [20].

It is possible, although unlikely, that the differences reported between WEHI-3B(D^+) and M1 cells reflect a difference in technique, as this study and those performed by Sachs and co-workers [16,17,44] have utilized only slight variations on O'Farrell's well-characterized 2D electrophoretic system [32]. It is possible that some differences may be due to differences in the protein stimulus used, as we used G-CSF partially purified from endotoxin serum, whereas Liebermann and Sachs [16] used unpurified endotoxin serum containing G-CSF and GM-CSF. Alternatively, the presence of C-type viral particles in M1 cells [47] may in some fashion amplify the number of G-CSF-induced protein events, as the presence of the viruses has been shown to vary during M1 cells' differentiation [24]. WEHI-3B(D^+) cells appear not to have any associated C-type viral particles [Dr. T.E. Mandel, personal communication].

The absence of MW markers or a numbering system on the 2D gels that are consistent among the various publications of Liebermann et al [16,23,24,44,47] have made direct comparisons with our data difficult. Aligning the patterns according to visual best fit showed that the acidic proteins (Figs. 1B, 2A–H) probably corresponded to the labile proteins 25, 26, 27, and 28 [16] which were similarly suppressed in response to MGI. Similarly, a large protein migrating identically to protein v1 was found to increase its rate of synthesis during MGI treatment [16]. Thus whilst there is concordance between the data for M1 and WEHI-3B cells in terms of several modulated proteins, the reason for the qualitative differences in the number of modulated proteins has yet to be determined.

The data obtained from WEHI-3B(D^+) cells induced to differentiate with G-CSF supports the hypothesis that constitutive synthesis of certain proteins may cause the "differentiation block" found in leukemic cells [45] or may be responsible for the maintenance of a transformed phenotype [22]. Decreased synthesis of these proteins (eg, by G-CSF treatment or by treatment with protein synthesis inhibitors [46]) may be the mechanism for overcoming these blocks. Similarly, the loss of the growth requirement for platelet-derived growth factor (PDGF) has been recently linked to the constitutive synthesis of two proteins in a transformed variant of 3T3 cells. The synthesis of these proteins is initiated in normal contact-inhibited 3T3 cells by treatment with PDGF, a process which overcomes the normal culture constraints on the cells [22].

The detection of a specific pattern of G-CSF-induced protein synthesis changes which have been localized to nuclear or cytoplasmic compartments (eg, during commitment of WEHI-3B(D^+) cells) should allow a more rational approach to the study of leukemic cell differentiation, particularly with regard to localizing any constitutive protein synthesis. Identification of these proteins allows the possibility of their isolation (by extraction from gels, for example) and thence to the raising of antibodies. Treatment of leukemic cells with a lipophilic derivative of the Fab portion of an antibody directed toward a G-CSF-suppressed protein (eg, 84, 67, 36, 17, 35a, etc), for example, might be expected to mimic the induction of G-CSF-induced differentiation by decreasing the cellular availability of the protein(s). Our data indicate that WEHI-3B(D^+) cells follow a reproducible program of protein synthesis and degradation during induction of differentiation. The initiation of cellular differentiation was rapid, and the concomitant protein changes occurring in the nucleus suggest that such proteins may play an important role in the conversion of leukemic cells to mature nondividing end cells. The identification of the subcellular location of these proteins should assist in their functional characterization.

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