Journal of Cellular Biochemistry 23:241–258 (1983)

Granulocyte Macrophage-Colony Stimulating Factor Stimulates the Synthesis of Membrane and Nuclear Proteins in Murine Neutrophils

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The effect of granulocyte macrophage-colony stimulating factor (GM-CSF), a well-characterized hemopoietic regulator, on protein synthesis in murine bone marrow neutrophils is described. Bone marrow neutrophils in excess of 95% purity were obtained by fluorescence-activated cell sorting. While GM-CSF did not appear to slow the rate of dying of peritoneal exudate neutrophils or thymus cells, the viability of bone marrow neutrophils after 17 hr was enhanced (40%) by GM-CSF. GM-CFS had no effect on total ^{35}S-methionine incorporation by thymocytes or peritoneal exudate neutrophils over a 17-hr incubation period; however, bone marrow neutrophils showed increased incorporation (approximately 10%) at all times between 5–17 hr. As viability and ^{35}S-methionine incorporation of bone marrow neutrophils at 5 hr were minimally affected by GM-CSF, this time point was chosen to study the effect of GM-CSF on the synthesis of particular proteins. Two-dimensional polyacrylamide gels of ^{35}S-methionine-labelled lysates were prepared from whole cells, isolated nuclei, and membranes. Quantitative analysis of the fluorograms obtained from the two-dimensional electropherograms by a computer-linked optical data digitiser indicated that out of a total of 180 proteins, the amount of label contained in 11 proteins was significantly higher in the presence of GM-CSF, while three proteins, apparently of cytoplasmic origin, contained less label than control cells. Eight of these proteins were identified as nuclear, and one was membrane derived. Attempts have been made to identify some of the inducible proteins and to correlate results with other studies of normal hemopoietic and leukemic cells. The significance and multiple functions of GM-CSF in hemopoiesis are discussed.

Key words: bone marrow neutrophils, gel electrophoresis (2-D), hemopoiesis, hemopoietic regulator, membrane proteins, nuclear proteins, optical data digitiser

Hemopoiesis is one of the few eukaryotic systems available for the detailed study of the molecular mechanisms controlling normal proliferation and differentiation [1,2]. The availability of semisolid agar culture techniques [3–5], purified committed progenitor cells (colony forming cells, CFC) [6,7], and well-characterised proteins (colony stimulating factors, CSF) [8,9] that control the proliferation and

Received March 10, 1983; revised and accepted September 23, 1983.

differentiation of CFC has made possible a detailed analysis of the proliferation kinetics and morphological maturation of CFC [8, 9, reviews]. In particular, purified granulocyte-macrophage colony stimulating factor (GM-CSF) [10] has been shown to facilitate the generation of mature granulocytes and macrophages in vivo [11] and in vitro [3,4,7]. Information on factors which stimulate self-replication and commitment of multipotential stem cells is limited, but evidence is accumulating that lineage-specific factors, such as GM-CSF, may act on multipotential precursors to generate clones of granulocyte and macrophage progenitors [12,13].

However, there is now increasing evidence that CSF is not merely a proliferative stimulus for immature cells, but also activates a number of functional properties in mature granulocytes and macrophages [14–18]. Thus GM-CSF stimulates RNA synthesis in bone marrow cells within 10 min [14] and DNA and protein synthesis within a few hours [15]. In macrophages, GM-CSF leads to increased phagocytosis and parasite killing [16] and increased synthesis of both plasminogen activator [17] and prostaglandin E [18].

Parallel investigations with a colony stimulating factor for cells in the mononuclear phagocytic series (CSF-1), which has been purified from mouse L-cells [19], have shown that CSF-1 also can perform the several functions of inducing precursors to proliferate [20], followed by maturation and functional activation of macrophages [21]. Additionally, however, CSF-1 appears to facilitate long-term (12 days) survival of macrophages without division or, in fact, induces mature macrophages to proliferate with a doubling time of 24 hr [22]. These latter phenomena are dependent on CSF-1 concentration and appear to be homeostatically regulated through selective destruction of CSF-1 by macrophages [22].

The immediate precursors for peritoneal exudate neutrophils (PEN) are generated in the bone marrow from CFC in a series of well-characterised steps [6,7]. In contrast to bone marrow macrophages [22], bone marrow neutrophils (BMN) are postmitotic; however, they continue to mature [23,24]. BMN and PEN are thus good models to study molecular changes accompanying GM-CSF-induced maturation and functional activation with no possible contribution from proliferative events [22]. Murine neutrophils constitute 45% of the nucleated bone marrow cell population, but, unlike PEN [25], bone marrow neutrophils (BMN) cannot be purified by density gradient centrifugation, and only the development of fluorescence-activated cell sorting has made it possible to obtain a 95% pure population [26].

To study GM-CSF-induced protein synthesis in BMN, total incorporation of ^{35}S-methionine was assayed, and specific changes in protein synthesis identified by two-dimensional gel electrophoresis and subsequent fluorography [27]. An optical data digitiser enabled quantification of all proteins synthesized [28]. Fractionation of radioactively labelled cells into nuclei and membrane components demonstrated for the first time that functional activation of neutrophils involved modulation of the synthesis or degradation of both nuclear and membrane proteins. The synthesis of different BMN protein has been compared with protein synthesis of more mature neutrophils (PEN), less mature normal myeloid progenitor cells (CFC), and leukemic cell lines.

MATERIALS AND METHODS

Materials

Acrylamide, N,N′-methylenebisacrylamide, Photoflo, and RP-5 X-Omat film were from Eastman Kodak. N,N,N′,N′-tetramethylethylenediamine, ammonium persulphate, PAGE Blue 83, and ethylenediamine tetraacetate (EDTA) were obtained

TABLE I: Optical Parameters for the FACS II Cell Sorter

Parameter	Laser wavelength (NM) 351.1/363.8	514.7
0° Scatter optical filter	ND 10	none
90° Scatter optical filter	560 EP	ND 2.5
Autofluorescence optical filter	450 WB	-

ND, neutral density filter; EP, low pass filter; WB, wide band selective filter.

from BDH. Tween-40, phenylmethylsulphonylfluoride (PMSF) and DNA ase I were obtained from Sigma. Urea was 'Ultra Pure' grade from Schwarz Mann. Triton-X 100 was from Packard. Fetal calf serum was from Flow Laboratories. Dulbecco's modified Eagle's (DME) medium and RPMI medium were obtained from Gibco. Methionine-free Dulbecco's modified Eagle's medium (DMEM) was obtained from Commonwealth Serum Laboratories, Poplar Road, Parkville. ^{35}S-methionine (1,200 Ci/mmol) was obtained from Amersham, England. All other chemicals used were from standard chemical sources of A.R. grade.

Experimental Animals

Mice used were male C57BL/6f/J mice (9 wk old), bred under specific pathogen-free conditions at the Walter and Eliza Hall Institute of Medical Research, Parkville, Australia, and conventionalized at 5 wk.

Isolation and Radioactive Labelling of Murine Bone Marrow Neutrophils

Bone marrow cells were separated using a fluorescence-activated cell sorter (FACS II) (Becton-Dickinson, Mountain View, CA) on the basis of their light-scattering and autofluorescent characteristics. The availability of a second laser enabled us to combine the two approaches described by Watt et al [26] using light in the visible region to analyse low- and high-angle scatter profiles and an excitation wavelength in the ultraviolet region to determine the autofluorescence distribution. Details of wavelengths and optical filters are shown in Table I.

Following a one-hr preincubation period, purified bone marrow neutrophils (10^6/ml) were biosynthetically labelled in methionine-free Dulbecco's modified Eagle's medium containing fetal calf serum (10%, v/v), ^{35}S-methionine (500μCi/ml), and the appropriate stimulus at supramaximal concentration. Labelling was carried out for 5 hr in a humidified atmosphere of 10% CO_2 at 37°C. The stimulus used was granulocyte-macrophage colony stimulating factor (GM-CSF), synthesized in vitro by lung tissue from C57BL mice preinjected with 5 μg endotoxin. The GM-CSF preparation used was stage V fractionation of the mouse lung conditioned medium (10^7 units/mg) [10], referred to as GM-CSF in the text. After labelling, the cells were diluted to 3 ml with phosphate-buffered saline, pH7.3 (PBS: sodium phosphate, 20 mM and NaCl, 149 mM) containing methionine (2 mM), and kept on ice for 30 min. A 20-fold excess of unlabelled bone marrow cells for whole cell lysate preparations and a 40-fold excess of thymocytes for membrane preparation were added, and the cells were washed four times with ice cold PBS.

Preparation of Protein Extracts for Electrophoresis

Extracts for two-dimensional protein mapping were prepared using a procedure modified from that described by O'Farrell [27]. The cell pellet was freeze-thawed

five times and the DNA digested with DNA'ase I (l mg/lml Tris-HC1 10 mM, pH7.4; 50% glycerol) in the presence of $MnCl_2$ (0.66 mM) and PMSF (10 mM), followed by EDTA (20 mM). SDS-lysis buffer, urea, and isoelectric focussing buffer were added as described by Watt et al [29]. Samples were centrifuged at 26 psi (110,000g) for 1 hr in a Beckman air-driven centrifuge (airfuge) prior to isoelectric focussing.

Preparation of Membrane and Nuclear Extracts

Membranes and nuclei were prepared from ^{35}S-methionine-labelled neutrophils with a 40-fold excess of unlabelled thymocytes using a procedure similar to that described by Standring and Williams [30]. The whole procedure was carried out on ice. Cells were suspended at 4 x 10^7/ml in Tris/saline (25 mM Tris-HC1, 150 mM Nacl, pH 7.4), an equal volume of Tris/saline containing 4% Tween-40 was added, and the mixture was stirred for 60 min in a Dounce homogenizer tube (7 ml) by adding a small magnetic stirring bar into the tube. After removing the stirring bar, the cell mixture was homogenized by six passes of a Dounce pestle B (loose fitting). Cells were checked at each homogenization step by eosin dye exclusion and phase contrast microscopy. Viability was reduced to 1% (eosin dye exclusion) and 97% of nuclei were free of cytoplasm (phase contrast microscopy). The homogenate was centrifuged at 260g for 15 min. The nuclear pellet was washed two times in Tris/saline pH 7.4, containing $MgSO_4$ (10 mM) and prepared for two-dimensional electrophoresis as described above for whole cells.

PMSF and EDTA were added at 10 and 20 mM, respectively, to the supernatant fluid before centrifugation at 3,000g for 30 min. The 3,000g pellet was discarded, and the supernatant fraction was airfuged for 1 hr at 28 psi (130,000g) at 4°C. The membrane pellet was washed once in Tris-HC1 (1 mM, pH 7.4). Lactate dehydrogenase activity was estimated [31] on a homogenate of whole PEN and PEN membranes.

Membrane Solubilization

The washed membrane pellet was prepared for isoelectric focussing using a procedure adapted for small samples from a method described by Ames and Nikaido [32]. Membranes from 2.5 x 10^7 cells (neutrophils and filler cells) were suspended in the airfuge tube in 15 μl solubilization buffer (50 mM Tris-HC1, pH 6.8, 2% sodium dodecyl sulfate [SDS], 0.5 mM $MgSO_4$, 10% 2-mercaptoethanol, 10 mM PMSF. The sealed tubes were incubated with shaking at 70°C for 1 hr. The solubilized membrane was diluted with 3 vol of sample dilution buffer (8 M urea, 5.4% Triton X-100, 5% LKB ampholines [4% pH 5–8, 1% pH 3.5–10], 5% 2-mercaptoethanol) and EDTA to 20 mM. The samples were airfuged at 26 psi (110,000g) for 1 hr prior to isoelectric focussing.

Isoelectric Focussing

The isoelectric focussing gel was prepared essentially as described by O'Farrell and O'Farrell [33], but gels (5.5 cm long) were poured in 100μl Micropet tubes (Clay Adams), pretreated with a 0.5% solution of Photoflo (Kodak). The complete protein extract was loaded in a volume of 25–40μl. After prefocussing samples at 50 V for 30 min, the gels were focussed at 500V for 4 hr, terminating with 1,000 V for 10 min. The pH gradient was determined with a Pharmacia isoelectric focussing kit in the pI range 3–10.

Discontinuous SDS-Polyacrylamide Gel Electrophoresis (SDS-Page)

Uniform concentration acrylamide (12%) [34] running gels (6 cm x 6 cm x 0.7 mm) were poured between two 8 x 8cm^2 glass plates.The isoelectric focussing gel was equilibrated for 15–20 min in SDS-sample buffer [33] (without 2-mercaptoethanol), placed midway into the stacking gel before this had polymerized, and electrophoresed in a Pharmacia GE 2/4 gel electrophoresis apparatus. Electrophoresis was performed at 20 mA/gel until the bromophenol blue marker reached the end of the slab. The gels were stained with PAGE Blue 83 (0.05% w/v in 50% v/v methanol, 10% v/v acetic acid) [35]. Standard molecular weight markers (Pharmacia Pty., Ltd.) were included in the gels. The gels were prepared for fluorography (Enhance, New England Nuclear) and exposed to preflashed Kodak RPX-Omat films [36]..

Estimation of Total Protein Synthesis

Casein-induced PEN from C57BL mice were prepared as previously described [25]. Single-cell suspensions of thymocytes were prepared by gently pushing a whole thymus, suspended in PBS at 4°C, through a fine stainless steel mesh with a glass pestle. Red blood cells were removed by resuspending the cells for 15 min at room temperature in ammonium chloride (0.168 M), then washing the thymocytes four times in PBS. BMN (prepared as described above), PEN, and thymocytes were suspended in 96-well Linbro microtitre trays at 34,000 cells/200μl in methionine-free DME containing 10% fetal calf serum. Following a preincubation for 1 hr at 37°C in a humidified atmosphere of 10% CO_2 in air, 20 μCi ^{35}S-methionine were added together with 7 x 10^3 units of GM-CSF or normal saline. Cells were incubated for 1,3,5, or 17 hr and harvested on a PHD Cell Harvesting System (Cambridge Technology). The cells were washed with normal saline, water-precipitated onto glass fibre paper, methanol-dried prior to being dissolved in 0.5 ml Ista-Gel (Packard), and counted on a LKB Wallace 1210 Ultrobeta scintillation counter.

Optical Data Digitiser (ODD)

The ODD system was designed to analyze autoradiographs and fluorographs of two-dimensional electropherograms in a semimanual, interactive mode [28]. The ODD consists of a Hammamatsu camera interfaced via DAOS [37] to a PDP-11/23 computer. The view of the film displayed on the monitor can be considered a 1,024 x 1,024 matrix of points. Individual protein spots are integrated by defining an area surrounding the spot and integrating by a series of linear scans within the defined region.

RESULTS

Purification of Bone Marrow Neutrophils

The use of a second laser in the FACS II system enabled cells to be exposed simultaneously to excitation wavelengths of 351.1/363.8 nm in the ultraviolet region, as well as 514.7 nm in the visible region (Table I) [26]. Cell separation could thus be improved by using light in the visible region for the analysis of low-angle and high-angle scatter distributions (Fig. 1), enabling a clearer separation of lymphocytes and neutrophils than had previously been achieved [26] (using the low-angle scatter parameter), and of the less mature neutrophils, monocytes, and lymphocytes from the

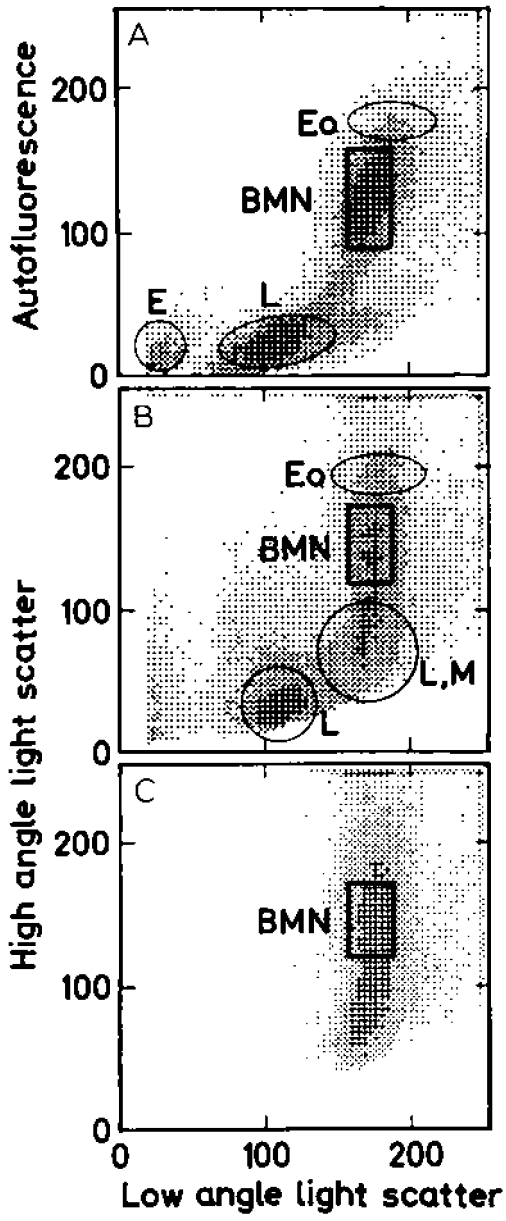

Fig. 1. Separation of bone marrow neutrophils from C57BL male mice using the FACS II fluorescence-activated cell sorter with laser wavelengths of 514.7 nm (low- and high-angle scatter) and 351.1/363.8 nm (autofluorescence). Two-dimensional contour representation of bone marrow cells following erythrocyte lysis plotted as a function of low-angle light scatter vs autofluorescence (A) or vs high-angle light scatter (B). C is as B after selecting the cells in the high-fluorescence window (marked BMN) in A. Areas of high cell density are black. Cells in the areas labelled BMN (bone marrow neutrophils), Eo (eosinophils), L (lymphocytes), M (macrophages), E (erythrocytes and dead cells) were collected for analysis.

more mature neutrophils (using the high-angle scatter parameter). Using these two parameters and retaining the fluorescence parameter described previously[26] allowed the isolation of mature neutrophils with ring-shaped, slightly condensed nuclei in excess of 97% purity, representing 11–15% of the nucleated bone marrow cell population and 24-32% of the total bone marrow neutrophil population.

Correlation of Total Protein Synthesis and Cell Viability

Figure 2 shows that protein synthesis as measured by ^{35}S-methionine incorporation into the proteins of GM-CSF-stimulated and control BMN increased over the first 5 hr. After that time, counts incorporated in the presence of GM-CSF remained at plateau level while counts in control BMN decreased to about 50% over the next 12 hr. This pattern is reflected in cell viability, which was 90% until the 5-hr time point for control and stimulated BMN. After 17 hr in the presence of GM-CSF, BMN showed 56% of maximal viability, while viability of control cells had dropped to 17% ($P < .001$). At 5 hr stimulated BMN had incorporated approximately 10% more counts than control cultures. PEN and thymocytes incorporated ^{35}S-methionine linearly during the first 5 hr. However, the presence of GM-CSF did not appear to stimulate increased protein synthesis, nor did it enable PEN or thymocytes to maintain plateau levels of ^{35}S-methionine incorporation. While GM-CSF had no effect on survival of thymocytes, it did somewhat enhance the viability of PEN over 17 hr ($.001 < P < .005$).

Preparation of Nuclei and Membranes

Following their incubation with ^{35}S-methionine, cells were either prepared for analysis by two-dimensional gel electrophoresis or processed in order to isolate their nuclei and plasma membranes so that the subcellular location of some proteins could be determined. Proteins from the cytoplasmic compartment were discarded.

The method used here to isolate nuclei and membranes was developed by Standring and Williams [30] for thymocytes. The suitability of this technique for neutrophils was tested with casein-induced peritoneal exudate neutrophils, which are easily obtained in large numbers. An analysis by eosin dye exclusion showed that cells were 65% viable following the Tween 20 treatment and 2% viable after three passes with the Dounce homogenizer pestle (Fig.3).Phase contrast microscopy showed that 97% of nuclei were free of cytoplasmic contamination after six passes with the Dounce homogeniser pestle (Fig. 3).Thus bone marrow derived neutrophils were homogenised six times.

A major problem in the preparation of membranes was the very low cell number ($0.5–1 \times 10^6$ BMN/incubation). To increase recovery, the labelled BMN cells were mixed with 40-fold excess (2×10^7) of unlabelled thymocytes. To avoid protein overloading of gels, it did not seem advisable to exceed this number of filler cells, as the low amount of ^{35}S-methionine incorporated by BMN necessitated loading the whole membrane extract during analysis by two-dimensional gel electrophoresis. Following the addition of protease inhibitors and the removal of the 3,000g pellet, membranes were collected by pelleting the 3,000g supernatant in several steps onto the same position in a Beckman airfuge tube (175 μl, 130,000g, 4°C). Attempts to purify the membranes using a discontinuous sucrose density gradient resulted in low yields. However, an estimation of lactate dehydrogenase activity, a cytoplasmic

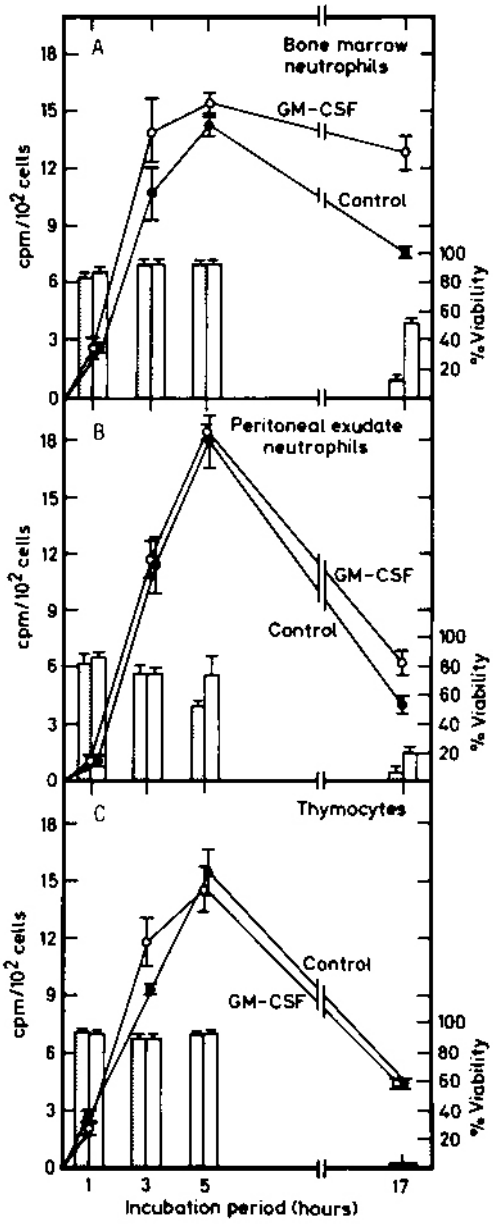

Fig. 2. Viability (□ ▨) and incorporation of ^{35}S-methionine (○ ●) in the absence (white symbols) and presence (dark symbols) of GM-CSF of bone marrow neutrophils (A), peritoneal exudate neutrophils (B), and thymocytes (C). Cells were incubated for 1, 3, 5, and 17 hr and harvested as described in Methods. Viability was estimated by eosin dye exclusion and incorporation of ^{35}S-methionine by scintillation counting. Standard errors of the mean are indicated.

enzyme marker [31,38], in the 130,000g airfuge pellet showed a low level of cytoplasmic contamination (1.65%) in the membrane preparation. Thus the 130,000g membrane pellet was used for electrophoretic analysis. Solubilization and extraction of proteins was carried out in the same airfuge tube, followed by a further centrifugation step prior to isoelectric focussing. This procedure gave reproducible two-dimensional protein maps in five separate experiments and minimized losses.

Fluorograms of Neutrophil Lysates

When ^{35}S-methionine-labelled protein extracts (3 x 10^4cpm approximately) from equal numbers of GM-CSF-stimulated and control bone marrow neutrophils were analyzed by two-dimensional electrophoresis, the fluorographs obtained following equal exposure periods revealed a reproducible pattern of about 180 proteins (Fig. 4) in three separate experiments. Careful examination of the fluorograms showed no qualitative protein changes following stimulation; however, as most of the radioactivity was associated with three proteins (actin, 14a, b), the low abundance proteins are barely visible in Figure 4. Following a quantitative computer analysis using an optical data digitiser (see below) two observations can be made: First, a general increase in the amount of most radiolabelled proteins can be demonstrated in the presence of GM-CSF. It is not certain whether this is due to an accelerated rate of protein synthesis or to inhibition of degradation of the newly synthesized proteins. But it is clear that the 10% increase in counts incorporated in the presence of GM-CSF (Fig. 2) is a result of a general rather than specific increase in labelled protein. Secondly, notwithstanding the general increase, 11 proteins out of 180 were found to be raised significantly above the level of the general increase, while three proteins were significantly reduced. In Figure 4, proteins are identified by their molecular weights. Proteins of the same molecular weight are distinguished by "a" and "b" according to

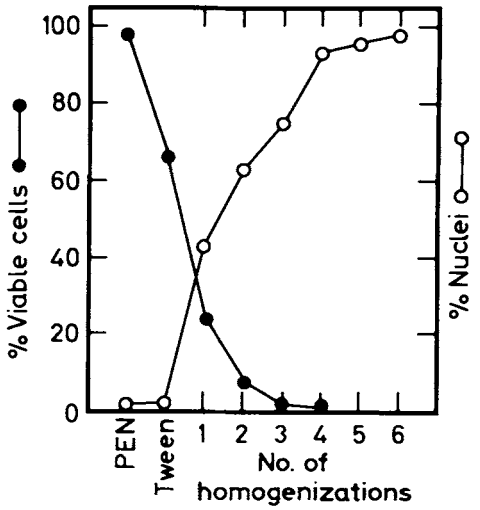

Fig. 3. The viability (-●-) of peritoneal exudate neutrophils was checked by eosin dye exclusion following collection (PEN), after 1 hr in 2% Tween 20, 4°C (Tween), and after each of six passes with a Dounce homogenizer pestle (1-6). The percentage of whole cells or nuclei with attached cytoplasmic fragments at each stage of treatment was monitored by phase contrast microscopy (-○-).

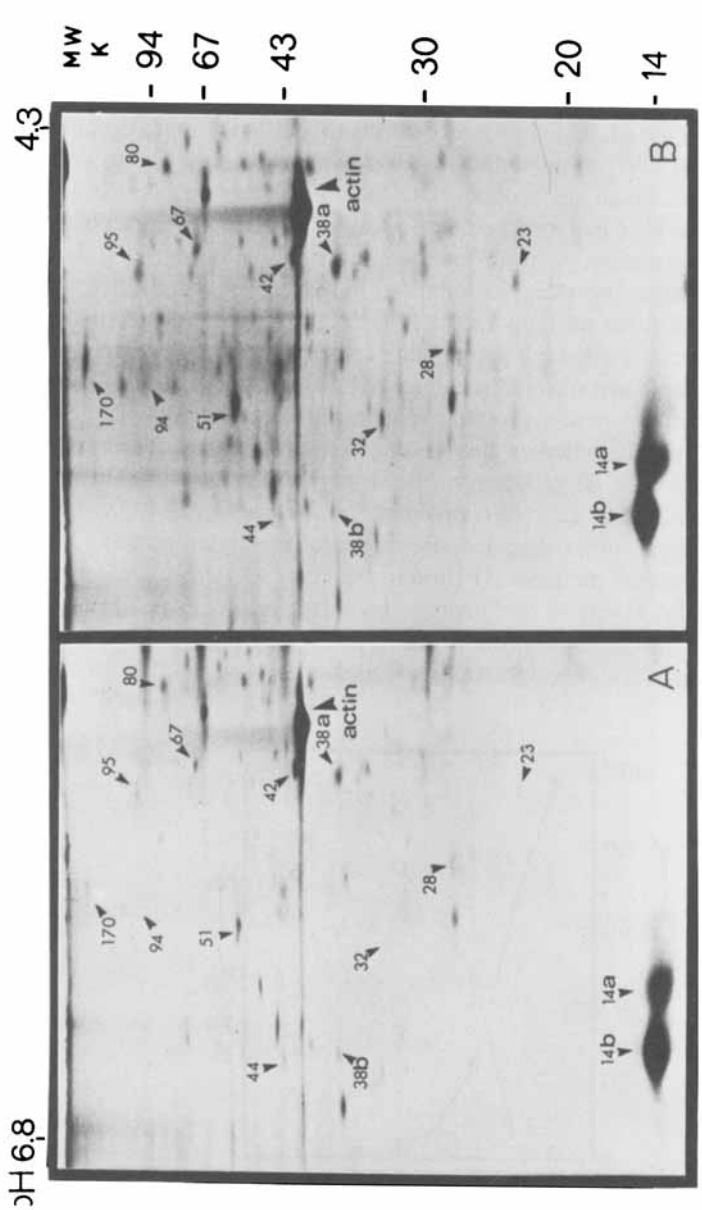

Fig. 4. Bone marrow neutrophils (2×10^5) were incubated with ^{35}S-methionine for 5 hr in the absence (A) and presence (B) of GM-CSF. Following extraction, the radiolabelled proteins (3×10^4cpm) were analyzed by two-dimensional isoelectric focussing/SDS - polyacrylamide gel electrophoresis and fluorographed for 3 mo. Molecular weights are marked in kilodaltons on the ordinate and pH units on the abscissa. The numerals used to define proteins are derived from their molecular weight x 10^{-3}. Similar fluorographs were obtained in three separate experiments.

their relative acidity or basicity. Thus increased levels are observed for proteins 14a, 23, 28, 32, 38a, 51, 67, 80, 94, 95, and 170 and decreased levels for proteins 14b, 38b and 44.

Computer Analysis by ODD of Fluorograms

The optical data digitiser (ODD) used for the quantitation of protein spots is a semimanual computerized scanning system [28]. To enable a comparison of the intensities of spots on fluorograms that are not perfectly superimposable, the ODD was designed to allow the rapid selection of the spots to be integrated. Integral values are linearly related and proportional to the optical densities of spots.

The correlation of the relative amounts of proteins synthesized by BMN in the presence and absence of GM-CSF is shown in Figure 5. A large range of integrated values was recorded from a low 220 to a high 250,000, necessitating representation on a log/log system. The correlation coefficient was 0.977. A regression line was fitted and the 99% confidence limits calculated. In Figure 5, points falling above the regression line represent proteins showing a GM-CSF-induced increase in protein synthesis and those below a decrease.The numbers identifying the points correspond to proteins in Figure 4. If label incorporation by stimulated and control cells were equivalent, the regression line (Fig. 5) would be linear and pass through the origin. The height of intersection of the regression line with the y-axis is an indicator of the overall increase in protein synthesis by GM-CSF stimulated cells.

The majority of proteins which fall outside the range defined by three standard deviations (proteins 14a, 28, 38a, 51,67, 80, 94, 95) were increased 1.2–3.5-fold or decreased (proteins 14b, 38b, 44) twofold in the presence of GM-CSF. Proteins 23, 32, and 170 were prominent, being increased 7.3-, 24.6-, and 17.5-fold, respectively, above control levels.

Analysis of Nuclear Proteins

Examination of two-dimensional fluorograms of nuclear protein extracts (Fig. 6) revealed many similarities to the maps of whole cell extracts. Actin, which has previously been shown to be a nuclear protein in WEHI-3BD+[39] and rat liver cells [40], was prominent among nuclear proteins. A high molecular weight (200 kilodalton) basic (pI 6.5) protein may tentatively be defined as myosin, as it displayed similar electrophoretic properties to a protein that Garrels [41] identified as the myosin heavy chain (MHC). Of the very highly induced proteins, both protein 23 and 32 were detected in nuclear extracts from stimulated cells, but not protein 170. Further, it appeared that GM-CSF-inducible proteins 28, 38a, 51, 80, 94, and 95 were also present in the nucleus. However, the three proteins whose synthesis was suppreseed — namely 16b, 38b, and 44 — were not detected in the nuclear lysates. A prominent protein of molecular weight 42,000 and pI similar to actin has also been shown to be of nuclear origin.

Analysis of Membrane Proteins

As cells interact with their environment through molecules contained in their membrane, it was very pertinent to examine the protein changes occurring in bone marrow neutrophils upon functional activation by GM-CSF. Upon activation, these cells are competent to follow a chemotactic gradient and to phagocytose. Fluorographs of proteins extracted from isolated membranes and subjected to analysis by

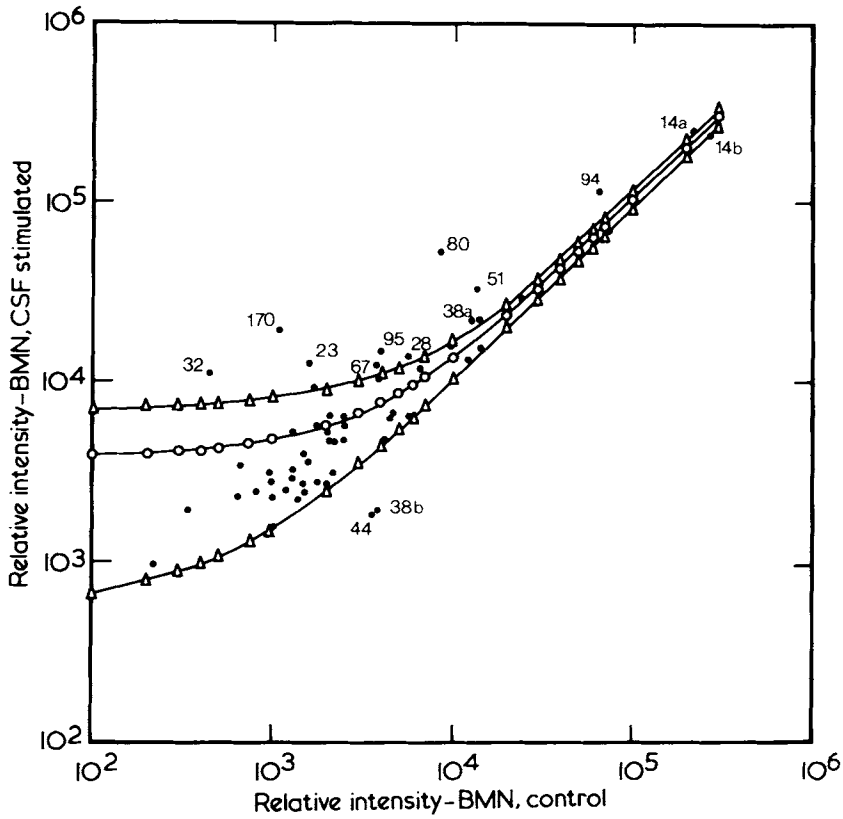

Fig. 5. The relative intensities of 56 matched spots from Figure 4A, B were determined by optical data digitiser (ODD) analysis and plotted as a scatter diagram of control protein vs the matching CSF-stimulated protein (●). Regression analysis gave a line of best fit (○), and the 99% confidence limits are shown (△). Statistical analysis was performed on a Hewlett-Packard HP41C pocket calculator.

two-dimensional gel electrophoresis are shown in Figure 7. As was also observed for whole cells and nuclei, the overall level of newly synthesized protein was higher in GM-CSF-stimulated cells. However, with the exception of actin, which was also a major membrane protein, and GM-CSF-induced protein 170, corresponding proteins could not easily be identified on protein maps from whole cells, presumably because of the relatively low abundance (1% of total) of membrane proteins. Several high molecular weight proteins that were not altered in the presence of GM-CSF were particularly abundant and were identified as strings of proteins of different pI and similar MW (eg, proteins 55, 69, 80; Fig. 7). Such strings of proteins may arise through differences in posttranslational glycosylation. Approximately 1.7 kilodalton/carbohydrate unit are added to the apparent molecular weight of proteins when analyzed by polyacrylamide gel electrophoresis [42]. A change in pI indicates the addition of a charged carbohydrate unit (eg, neuraminic acid).

Strings of apparently related proteins were also present below MW 40,000, several of them taking on a curvilinear configuration such that a decrease in pI

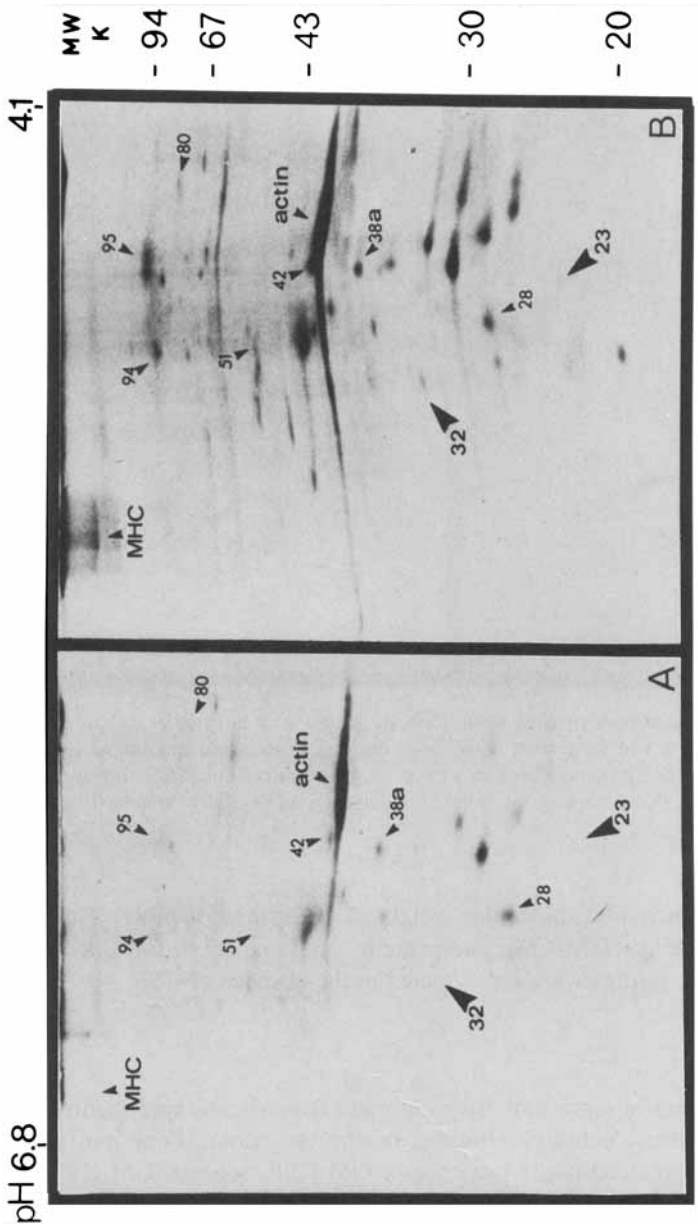

Fig. 6. ^{35}S-methionine-labelled proteins from isolated nuclei (5×10^5) were separated and analyzed as in Figure 4. Fluorographs were exposed for 2 mo. Nuclei were isolated from control BMN (A) and BMN incubated for 5 hr with GM-CSF (B). MHC, myosin heavy chain. The fluorographs reproduced here are typical of five separate experiments.

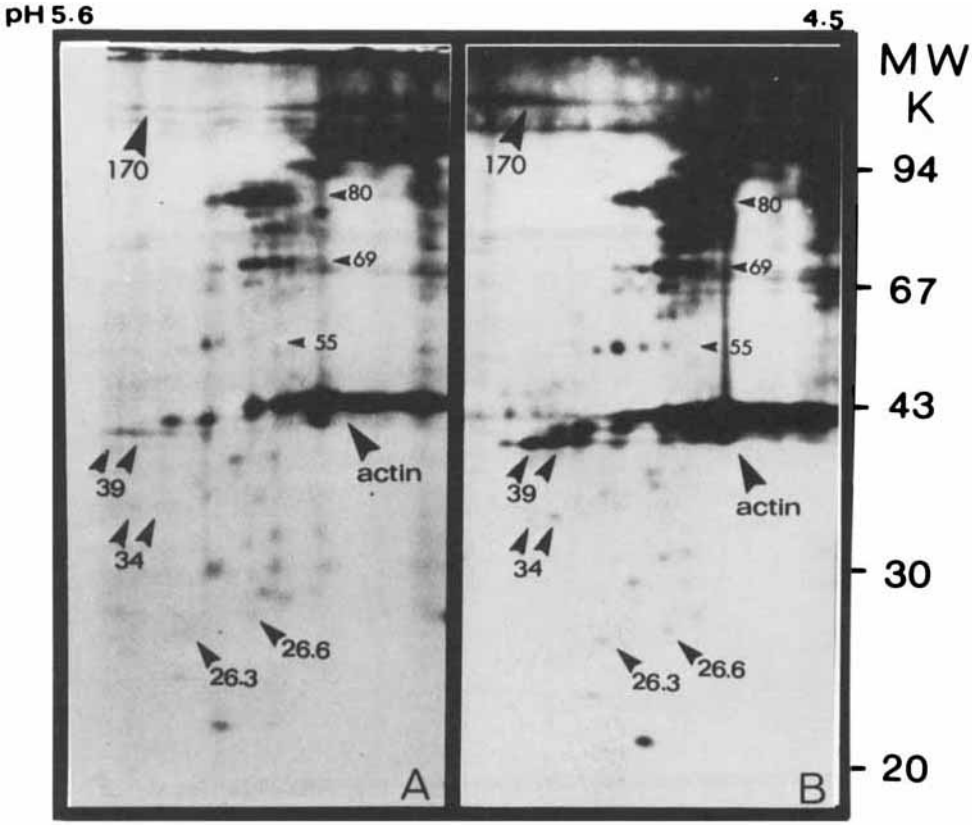

Fig. 7. The membranes of bone marrow neutrophils (6×10^5) were purified as described in Materials and Methods following a 5-hr incubation with ^{35}S-methionine in the absence (A) and presence (B) of GM-CSF. The radiolabelled proteins were extracted prior to two-dimensional electrophoretic analysis as in Figure 4. Gels were fluorographed for 3 mo. Similar fluorographs were obtained in five separate experiments.

coincides with an increased molecular weight. Two strings of proteins (proteins 39 and 34) were induced by GM-CSF, and proteins 26.3 and 26.6, forming part of two different curvilinear strings were not detected in the absence of CSF.

DISCUSSION

Three important observations have emerged from these experiments. First, the overall protein synthetic activity of mature postmitotic mouse bone marrow neutrophils was slightly enhanced in the presence of GM-CSF; second, GM-CSF appeared to slow the death rate of BMN over long periods; and third, the synthesis of a number of nuclear and membrane proteins appeared to be particularly sensitive to the presence of GM-CSF.

The present results have shown that when protein synthesis reached plateau levels at 5 hr, GM-CSF-stimulated neutrophils had incorporated about 10% more ^{35}S-methionine than control cultures. This plateau level was maintained for the following

12 hr in the continual presence of GM-CSF but fell to about 50% of peak levels in the absence of stimulus. It remains to be determined whether GM-CSF specifically enhanced methionine uptake, slowed down degradation of newly synthesized protein, or enabled cells to maintain a dynamic equilibrium of degradation and synthesis. Evidence [43] obtained with myeloid progenitor cells (colony forming cells) has suggested that protein degradation may be greatly reduced in the presence of CSF. However, Cooper and Burgess [39], investigating the opposite phenomenon — namely, decreased incorporation of radiolabel when a myeloid cell line (WEHI-3BD+) was induced to differentiate — report that both inhibition of protein synthesis and specific degradation contribute. Additional protein synthesis or maintenance of synthesized protein at plateau level were not observed in peritoneal exudate neutrophils nor in thymocytes, which are not considered to be GM-CSF target cells. The lack of effect on peritoneal exudate neutrophils may indicate that functional activation of neutrophils by GM-CSF is a once-only event occurring in the bone marrow. PEN, having been activated prior to their departure from the bone marrow, are therefore no longer susceptible to such stimulation.

While addressing the intriguing question as to why GM-CSF enhances the longevity of neutrophils in vitro (both bone marrow derived and from peritoneal exudates), the effect of CSF on three different types of target cell — namely, hemopoietic precursors (CFC), mature neutrophils (BMN) and myeloid leukemic cells (WEHI-3BD+) — must be considered. The continued presence of GM-CSF is required by immature granulocyte-macrophage progenitors (CFC), a type of cell that undergoes a burst of proliferation over a period of 5 days, with a doubling time of 13–18 hr [7]. While a population of CFC, deprived of CSF for 20 hr, will not contain any viable cells, it has not yet been possible to determine if CSF acts as a "survival" factor on a population of cells which, once triggered, are genetically programmed to divide a certain number of times followed and accompanied by biochemical and morphological changes culminating in the mature neutrophil or macrophage.

By contrast, WEHI-3BD+, a murine myelocytic leukemia line which resembles CFC morphologically, survives and divides in the absence of CSF. Indeed exposure to GM-CSF [44,45] can drive these cells towards maturation, perhaps analogously to the fate of CFC. Bone marrow neutrophils are postmitotic. The presence of GM-CSF enhances the cells' functional ability, eg, phagocytosis of bacteria [unpublished data], and retards but does not halt their ultimate death. Thus CSF can influence such diverse functions as 1) burst of proliferation, 2) progress to maturation, 3) functional activation, 4) enhanced survival. While no direct experimental evidence exists, this diversity of effects might argue for a genetically preprogrammed system where cells in that lineage display CSF receptors. Following the interaction with CSF, certain cellular changes occur which result in the appearance of CSF receptors triggering different cellular changes. Such models have been proposed for embryonic development [46].

While Figure 2 emphasizes that viability and ^{35}S-methionine incorporation are relatively poor parameters for measuring the early effects of GM-CSF on bone marrow neutrophils, an alteration in the rate of synthesis or degradation of individual proteins may be more indicative of the cells' response. It was convenient to prepare and analyze extracts from whole cells, isolated nuclei, and membranes, while, using the techniques described above, cytoplasmic proteins were too dilute to analyze in our system. We assumed tentatively that those proteins in whole cell lysates, which

were not present in nuclear or membrane extracts, were of cytoplasmic origin . Of the proteins whose synthesis was affected by GM-CSF, only two proteins (actin and protein 170) could be identified as membrane proteins, while eight proteins (proteins 23, 28, 32, 38a, 51, 80, 94, 95) were apparently of nuclear origin.

It is worth noting that, while two-dimensional gel electrophoresis is suited to monitor cellular protein changes, it is often hazardous to compare two-dimensional electropherograms obtained by different investigators. Different protein patterns result from variations in technique, and in the absence of a reference grid provided by molecular weight and pI markers it is difficult to make valid comparisons. Thus, we cannot with confidence cross-reference the neutrophilic proteins described here with protein changes induced in certain murine myeloid leukemic lines [47, 48] or human granulocytes from patients with myelogenous leukemia [49].

Following their release from the bone marrow in vivo, the nuclei of murine neutrophils become characteristically highly segmented, and a similar change is observed during in vitro incubation. An analysis of the protein changes accompanying such a drastic morphological change was therefore of interest. Previous investigations of human neutrophils have demonstrated the presence of dense interchromatinic granules, rich in RNA and nonhistone proteins [50]. This study has defined a number of GM-CSF-induced changes in nonhistone nuclear proteins. Whether these proteins participate in the segmentation of the nucleus remains to be determined. Actin and myosin are possible candidates for this function.

We have demonstrated that GM-CSF-induced changes in neutrophilic membrane proteins may be of two types: modification of the glycosylation pattern of newly synthesized proteins (eg, proteins 26.3,26.6) and enhanced synthesis of certain proteins (eg, actin, proteins 34, 39, 170). Of these, actin and protein 170 proved to be of particular interest. The involvement of actin in phagocytosis by rabbit pulmonary macrophages has previously been demonstrated [51, 52]. Although there are no corresponding studies with neutrophils, the report of a patient with recurrent bacterial infections, neutrophil actin dysfunction, and impaired phagocytosis [53] suggests a function for actin in the phagocytic process. While actin appears to be present in myeloid leukemic cell lines [39,47,48] the specific induction of membrane-associated actin concomitant with induced maturation has not previously been examined.

The identification of cell surface proteins and their functions in human and murine myeloid cells is increasingly facilitated by the use of monoclonal antibodies specifically mediating [54] or blocking [55] complement-dependent cytotoxicity, inhibiting chemotaxis [56] and neutrophil degranulation [57]. The inducible neutrophil protein of molecular weight 170 kilodaltons may be identical with the α-subunit of Mac-1, a protein identified by monoclonal antibody M1/70 [58,59]. It has been shown that complement-receptor-mediated rosetting of erythrocytes in murine neutrophils and macrophages is blocked by the anti-Mac-1 monoclonal antibody [55], and evidence [reviewed in 60] is accumulating that Mac-1 may be identical with the type 3 complement receptor (CR3). Despite thorough investigations [60] and purification [61] of Mac-1, it appears that no two-dimensional PAGE maps are presently available for this molecule. A molecule of similar electrophoretic behaviour to protein 170 appears to be synthesized de novo by WEHI-3BD+ cells following incubation with MGI for 6 days [48]. Two-dimensional autoradiographs of the iodinated surface proteins of peritoneal exudate neutrophils [29] show a prominent spot in the position

expected for protein 170. Experiments are presently underway to investigate the possible identity of protein 170 and Mac-1.

While differentiation markers are frequently synonymous with cell surface proteins defined by monoclonal antibodies, it appears that protein 42 is a nuclear differentiation marker. In the path from immature colony forming cell [43] to peritoneal exudate neutrophil, synthesis of protein 42 has been observed only in bone marrow neutrophils. Peritoneal exudate neutrophils, however, appear to maintain high levels of this protein (visualized by Coomassie staining) [29]. The role of this protein in the fate of the nucleus remains to be determined.

ACKNOWLEDGMENTS

We gratefully acknowledge assistance from Dr F.L. Battye with the fluorescence-activated cell sorter and from Drs G.C. Malachowski and P.C. Cooper with the optical data digitizer. Dr Don Metcalf is thanked for his critical comments during the preparation of the manuscript. We are grateful to Ms S. Blackford for help in the preparation of the manuscript.

I.J.S. receives an Australian Commonwealth Postgraduate Research Award, and the work at the Walter and Eliza Hall Institute of Medical Researcgh was supported by NIH grant No. CA-22556.

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