*Journal of Biochemical and Biophysical Methods*, 21 (1990) 47–58
Elsevier



JBBM 00819

# Analysis by high-resolution two-dimensional electrophoresis of differentiation-dependent alterations in cytosolic protein pattern of HL-60 leukemic cells

Peter R. Jungblut [1] and Roland Seifert [2]

[1] *Institut für Toxikologie und Embryonalpharmakologie, Institut für Humangenetik, Berlin and* [2] *Institut für Pharmakologie, Freie Universität Berlin, Berlin 33, F.R.G.*

(Received 23 December 1989)
(Revision accepted 15 February 1990)

## Summary

HL-60 leukemic cells were differentiated along the neutrophilic pathway with retinoic acid (RA) or along the monocytic pathway with 1,25-dihydroxyvitamin $D_3$ ($1,25(OH)_2D_3$). Using a high-resolution two-dimensional electrophoresis technique and subsequent silver staining, differentiation-dependent changes in cytosolic protein pattern of HL-60 cells were analysed and were compared with the cytosolic protein pattern of human neutrophils. The amount of 64 and 50 out of a total of 632 proteins studied was increased or decreased in RA- and $1,25(OH)_2D_3$-differentiated HL-60 cells, respectively, in comparison to undifferentiated HL-60 cells. Thirty-three of these proteins were similarly altered in RA- and $1,25(OH)_2D_3$-differentiated HL-60 cells. Twenty-two and 25 of the proteins altered in amount in RA- or $1,25(OH)_2D_3$-differentiated HL-60 cells versus undifferentiated HL-60 cells were similarly altered in human neutrophils in comparison to undifferentiated HL-60 cells. Seven and 10 of the proteins altered in amount in RA- or $1,25(OH)_2D_3$-differentiated HL-60 cells had specific equivalents in neutrophil cytosol. Our results show (i) that neutrophilic and monocytic differentiation is associated with decreases and increases in amount of cytosolic proteins; (ii) that both differentiation processes share a common set of alterations; and (iii) are associated with specific alterations in protein amount.

Key words: Two-dimensional electrophoresis; Silver staining; Differentiation; Retinoic acid; 1,25-dihydroxyvitamin $D_3$; Human neutrophils

## Introduction

The human leukemic HL-60 cell line is a useful model system for studying differentiation-dependent processes of phagocytes. Undifferentiated HL-60 cells are

*Correspondence address*: P.R. Jungblut, Institut für Toxikologie und Embryonalpharmakologie, Institut für Humangenetik, Freie Universität Berlin, Garystrasse 5, D-1000 Berlin 33, F.R.G.

pluripotent promyelocytes which are differentiated into neutrophil-like cells by dibutyryl cyclic AMP, dimethyl sulfoxide or retinoic acid (RA) and into monocyte-like cells by 1,25-dihydroxyvitamin $D_3$ ($1,25(OH)_2D_3$) [1–7]. Differentiation of HL-60 cells along the neutrophilic and monocytic pathway is associated with various biochemical changes such as an increase in hexose monophosphate shunt activity and NADPH oxidase activity, which catalyses superoxide formation [1–7]. In addition, the pattern of whole cell extract proteins, cytoskeletal proteins and phosphoproteins changes during differentiation as revealed by two-dimensional electrophoresis (2-DE) and radiochemical detection methods [8–10]. These results prompted us to analyse cytosolic proteins of RA- and $1,25(OH)_2D_3$-differentiated HL-60 cells by 2-DE and silver staining in order to further characterize these differentiation processes. Here we report on differential changes in amount of cytosolic proteins during neutrophilic and monocytic differentiation of HL-60 cells.

## Materials and Methods

*Materials*

Urea for gel solutions, overlay and underlay solutions and sample preparation, acrylamide and bisacrylamide for the first dimension, *N,N,N′,N′*-tetramethylethylenediamine (TEMED), ammonium persulfate (AP) and agarose were obtained from Bio-Rad (Richmond, CA). Urea for electrode solutions of the first dimension, glycerol, orthophosphoric acid, ethylenediamine, glycine and reagents for silver staining, except for thimerosal, were purchased from Merck (Darmstadt, F.R.G.). Acrylamide and bisacrylamide (Bis) for the second dimension, Servalyt ampholytes pH 5–7, Isodalt ampholytes pH 3–10, sodium dodecyl sulfate (SDS) and Triton X-100 were obtained from Serva (Heidelberg, F.R.G.). The experiments of our present study were performed with one single batch of Isodalt ampholytes pH 3–10. Tris base, Tris hydrochloride, $\beta$-mercaptoethanol, thimerosal and RA were purchased from Sigma (Taufkirchen, F.R.G.). Sephadex G200 Superfine was obtained from Pharmacia-LKB (Uppsala, Sweden). $1,25(OH)_2D_3$ was a kind gift of Drs. H. Gutmann and U. Fischer of Hoffmann-La Roche (Basel, Switzerland). Sources for cell culture media have been described elsewhere [1–3].

*Cell culture and cell fractionation*

HL-60 cells were cultured in suspension culture in RPMI-1640 medium supplemented with 10% (v/v) horse serum, 1% (v/v) non-essential amino acids, 2 mM L-glutamine, 50 U/ml penicillin and 50 $\mu$g/ml streptomycin in a humidified atmosphere with 7% $CO_2$ at 37 °C. For inducing neutrophilic or monocytic differentiation, HL-60 cells were seeded at $10^6$ cells/ml and were cultured for 168 h with 10 nM RA or for 120 h with 10 nM $1,25(OH)_2D_3$ [3,4,7]. Cell viability after differentiation was 90–95% as revealed by trypan blue dye exclusion. HL-60 cells were disrupted by nitrogen cavitation and cytosol was prepared as described [1,2]. Buffy coat preparations were obtained from the local blood bank. Human neutrophils were isolated by dextran sedimentation and centrifugation through Ficoll-

Hypaque. Cell preparations consisted of more than 98% neutrophils [11]. Neutrophils were disrupted by nitrogen cavitation and cytosol was prepared as described [11]. Protein determination was performed according to Lowry et al., using bovine serum albumin as standard [12].

*Two-dimensional electrophoresis*

2-DE was performed as described by Klose [13] with major modifications in order to improve practicability, sensitivity, resolution and reproducibility. Briefly, gel solutions containing all components of the gel except AP were prepared. In order to obtain a high degree of reproducibility, gels for the present investigation were prepared with one lot of each gel solution. The gel solutions for first dimension separation gel, cap gel and second dimension separation gel were stored at −70°C in aliquots of 2.0, 1.0, and 18.0 ml, respectively. After a storage time of at least 3 months we did not observe any loss of quality of the gel solutions. Longer storage times have not been investigated.

The chamber for the first dimension was home-made and consisted of two reservoirs to be filled with 1 liter of electrode solution each. The distance between the electrodes amounted to 15 cm. Eight glass tubes (outer diameter 6.0 mm, inner diameter 1.5 mm, length 9.3 cm) (Schott, Hofheim, FRG) with calibration rings at 3 and 9 mm from the lower end and at 13 mm from the upper end were inserted into the chamber. Glass tubes were closed and were filled with underlay solution (9 M urea, 20% (w/v) glycerol) from the lower end of the tube up to the 9 mm calibration ring. The separation gel solution for the first dimension was thawed, and after degassing 51.3 µl of a solution containing 0.8% (w/v) AP were added. After addition of AP the separation gel was rapidly filled into the tube up to the 13 mm calibration ring from the upper end. The resulting polymerized gel had a length of 7.1 cm and consisted of 9 M urea, 5% (w/v) glycerol, 4% (w/v) acrylamide, 0.3% (w/v) Bis (total percentage concentration (w/v) of acrylamide plus Bis ([T]) = 4.3%; percentage concentration (w/w) of Bis to the total concentration [T] ([C]) = 7.0%), 0.06% (v/v) TEMED, 1.33% (w/v) Servalyt ampholytes pH 5–7, 0.67% (w/v) Serva Isodalt ampholytes pH 3–10 and 0.02% (w/v) AP. The gel was overlayed with deionized water which was replaced by overlay solution consisting of 5 M urea, 5% (w/v) glycerol, 1.33% (w/v) Servalyt ampholytes pH 5–7, and 0.67% (w/v) Serva Isodalt ampholytes pH 3–10 after 30 min. The tubes were turned around and the underlay solution was removed. After degassing, 975 µl of the gel solution for the cap gel were mixed with 25 µl of a solution containing 0.8% (w/v) AP. The resulting polymerized cap gel consisted of 9 M urea, 5% (w/v) glycerol, 10% (w/v) acrylamide, 0.13% (w/v) Bis ([T] = 10.13%, [C] = 1.3%), 0.06% (v/v) TEMED, 0.67% (w/v) Servalyt ampholytes pH 5–7, 0.33% (w/v) Serva Isodalt ampholytes pH 3–10 and 0.02% (w/v) AP. Immediately after the addition of AP, the cap gel was filled into the tubes up to the 3 mm calibration ring and was overlayed with deionized water. The tubes were closed and stored at room temperature overnight.

After removing the water from the top of the cap gel and the overlay solution from the top of the separation gel the glass tube was filled up on the cap gel side with cathode solution containing 9 M urea, 5% (w/v) glycerol and 5% (v/v)

ethylenediamine. A Sephadex G200 gel with a column height of 2 mm was applied onto the separation gel. The Sephadex gel was prepared in 25% (w/v) glycerol and was stored at −70°C for up to 5 month. Directly prior to the application onto the separation gel, 270 mg of the Sephadex gel were mixed with 270 mg of urea, 25 μl of β-mercaptoethanol and 12.5 μl of ampholytes pH 5–7. Acetone precipitates of cytosolic proteins were dissolved in a solution consisting of 9 M urea, 4% (w/v) Triton X-100, 2% (v/v) β-mercaptoethanol, 1.33% (w/v) Servalyt pH 5–7, 0.67% (w/v) Serva Isodalt pH 3–10 and 12.6% (v/v) glycerol. Twenty μg of protein (10 μl) were applied onto the Sephadex gel. Thereafter, overlay solution was placed onto the sample with a column height of about 2 mm and the tube was filled up with anode solution containing 3 M urea and 742 mM phosphoric acid. One liter of anode solution was filled into the upper reservoir of the chamber. Isoelectric focusing was performed under non-equilibrium conditions [14]. Gels were run for 1 h each at 100, 200, 400 band 600 V; for 10 min at 800 V and for 5 min at 1000 V resulting in 1517 Vhs, using an LKB 2197 power supply. Preliminary results showed that these conditions were optimal with respect to protein spot resolution (data not shown).

Focused rod gels were extruded from the cap gel end by a polypropylene string. The sample application site of the gel was washed with deionized water to remove phosphoric acid of the anode solution. Thereafter the gels were incubated for 10 min in 10 ml of a solution consisting of 125 mM Tris-phosphate, pH 6.8, 40% (w/v) glycerol and 3% (w/v) SDS. Focused gels were stored at −70°C up to 3 weeks without loss of quality in the resulting gels. Longer storage times have not been investigated.

SDS gel electrophoresis was performed according to Laemmli [15] with modifications, using a Mini Protean II cell (Bio-Rad). Eighteen ml of separation gel solution for the second dimension were mixed with 1.2 ml of a solution containing 1.28% (w/v) AP. This mixture was poured between the glass plates. The resulting polymerized gel consisted of 0.375 M Tris-HCl, pH 8.8, 0.03% (v/v) TEMED, 15% (w/v) acrylamide, 0.2% (w/v) Bis ([T] = 15.2%, [C] = 1.3%), 0.1% (w/v) SDS and 0.08% (w/v) AP and had a size of 6.5 × 8.0 × 0.15 cm. The gel was overlayed with deionized water and was used after 1 h of polymerization at room temperature.

After removal of water, rod gels were applied onto the top of slab gels and were embedded in agarose solution consisting of 1% (w/v) agarose, 125 mM Tris-phosphate, pH 6.8, and 0.1% (w/v) SDS. The electrode buffer consisted of 25 mM Tris, 192 mM glycine and 0.1% (w/v) SDS. The gels were run for 5 min at 35 V, for 10 min at 55 V, for 15 min at 100 V and for 1 h at 150 V, using an LKB 2197 power supply. Thereafter, gels were incubated overnight in fixation solution consisting of 50% (v/v) methanol and 10% (v/v) acetic acid.

Silver staining of proteins was performed as described by Merril et al. [16] and Heukeshoven and Dernick [17] with modifications in order to obtain a penetrating silver stain without background staining. Gels were incubated for 2 h in a solution consisting of 30% (v/v) ethanol, 0.5 M sodium acetate, 0.5% (v/v) glutaraldehyde and 0.2% (w/v) sodium thiosulfate. Thereafter, gels were washed three times for 20 min in deionized water. Gels were impregnated for 30 min in a solution consisting

of 0.1% (w/v) silver nitrate and 0.01% (v/v) formaldehyde, followed by rinsing of the gels for 20 s in deionized water. Gels were developed for 25 min at room temperature in a solution containing 2.5% (w/v) sodium carbonate and 0.01% (v/v) formaldehyde, pH 10.9. Development of the gels was stopped by placing them into a solution containing 50 mM EDTA. Silver-stained gels were stored at 4°C in the dark in a solution consisting of 50 mM EDTA and 0.02% (w/v) thimerosal.

The distance between two spots in the first dimensional and second dimensional direction was assessed. The distance between the two spots in first dimension amounted to $66.8 \pm 1.5$ mm (standard deviation (SD), $n = 10$), and in the second dimension, the distance amounted to $58.7 \pm 1.1$ mm (SD, $n = 10$). This does not compare too badly with the reproducibility of spot position in 2-DE in immobilized pH gradients [21]. Protein patterns were analysed visually as described [18]. This visual evaluation reproducibly detects differences in the intensity of protein spots greater than 45% [19]. Prerequisites of this visual evaluation are to avoid misinterpretations due to biological variation of the samples, due to variation in the performance of gel electrophoresis and due to variation in the analysis of protein patterns by various investigators. In our present study, two different cytosol preparations of each cell type were analysed, and with each preparation at least three runs were performed. Differences in protein spot intensity which were reproducibly present in all gels analysed and were detected by two experienced investigators are marked with arrows and arrow heads in the gels shown in Fig. 1. As the spot composition of the protein patterns analysed was similar, most of the spots served as internal marker proteins for matching. Gels were calibrated with a mixture of proteins containing horse myoglobin, soy bean trypsin inhibitor, rabbit glyceraldehyde-3-phosphate dehydrogenase, rabbit phosphorylase *b*, bovine serum albumin (Sigma); bovine carbonic anhydrase (Serva) and aspartate aminotransferase from *E. coli* (a kind gift of Dr. M. Herold, Biozentrum, Basel, Switzerland).

## Results

Cytosolic protein patterns of undifferentiated HL-60 cells, of RA- and $1,25(OH)_2D_3$-differentiated HL-60 cells and of human neutrophils were compared (Figs. 1a–d). The high reproducibility of gels is shown in pars pro toto by the similarity of the positions of protein spots not marked with arrows or arrow heads in Figs. 1a, c and d. These proteins were not differentiation-dependently altered in amount in HL-60 cells. Protein patterns were compared and reproducible differences in protein spot intensity are marked with arrows and arrow heads in Fig. 1. Table 1 summarizes the differentiation-dependent alterations in intensity of cytosolic protein spots of HL-60 cells and compares them with the corresponding protein spots in human neutrophils, identical amounts of loaded protein being the basis of the comparison.

Among 632 proteins analysed, the amount of 64 proteins was increased or decreased in cytosol of RA-differentiated HL-60 cells in comparison to undifferentiated cells (arrows and arrow heads in Fig. 1c). Differentiation with RA was

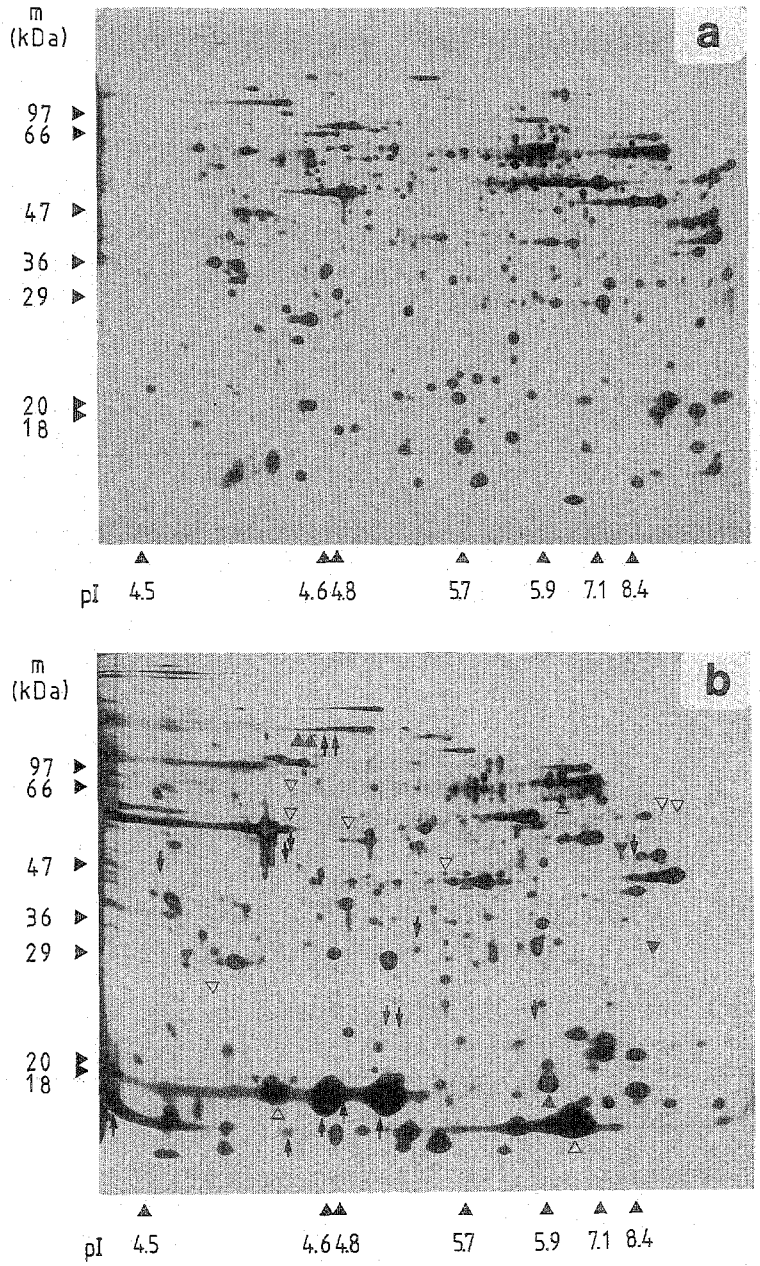
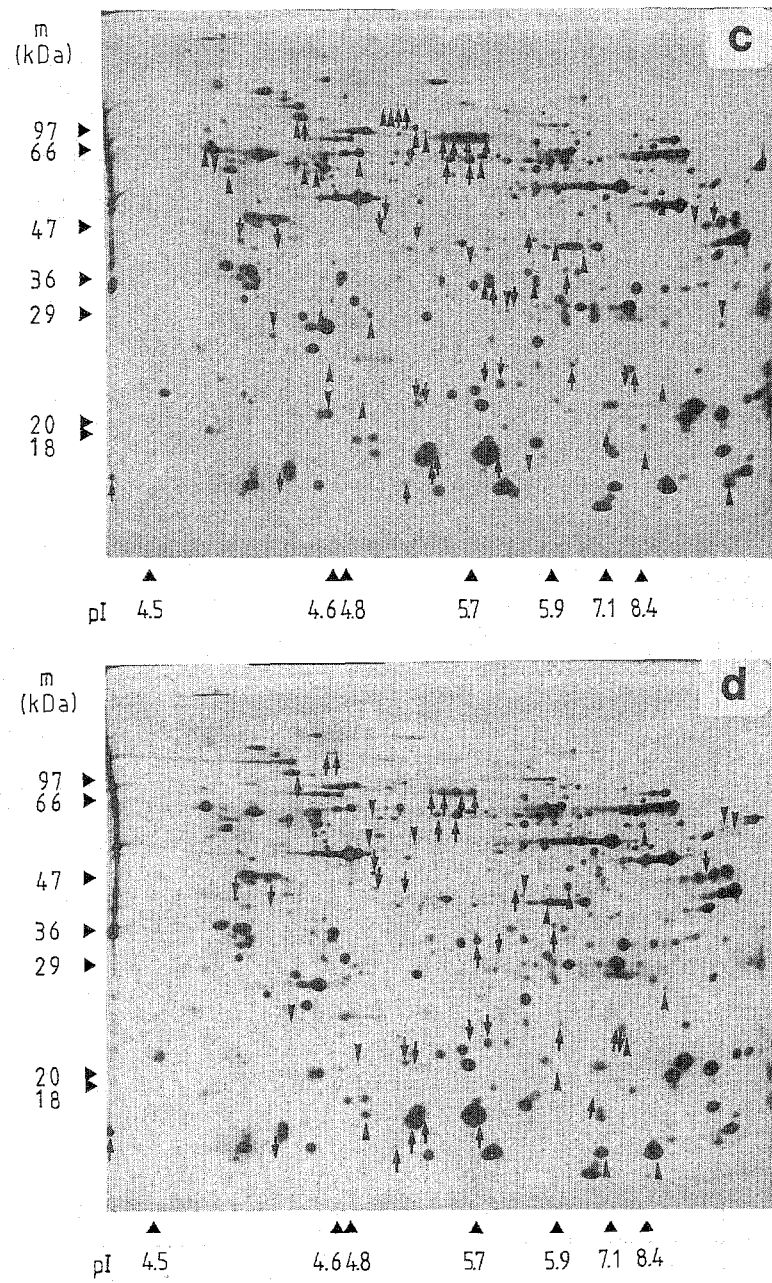



Fig. 1. 2-DE pattern of cytosolic proteins of HL-60 cells and human neutrophils. For isoelectric focusing, 20 $\mu$g of cytosolic protein of HL-60 cells or human neutrophils were applied at the anode of rod gels. For the second dimension, rod gels were applied at the top of SDS gels. SDS gels were stained with silver. (a) undifferentiated HL-60 cells; (b) human neutrophils; (c) RA-differentiated HL-60 cells; (d) $1,25(OH)_2D_3$-differentiated HL-60 cells. Representative gels are shown. Runs with samples of each cytosol were repeated three times and were highly reproducible. Analysis of cytosols from different preparations of HL-60 cells and human neutrophils also revealed a high degree of reproducibility. Differences in spot intensity present in all gels analysed are marked with arrows or arrow heads (see Materials and Methods). In b, spots being similarly altered in RA-differentiated HL-60 cells and




neutrophils in comparison to undifferentiated HL-60 cells are marked with closed arrow heads. Spots being similarly altered in $1,25(OH)_2D_3$-differentiated HL-60 cells and neutrophils in comparison to undifferentiated HL-60 cells are marked with open arrow heads. Spots being similarly altered in both RA and $1,25(OH)_2D_3$-differentiated HL-60 cells and neutrophils in comparison to undifferentiated HL-60 cells are marked with arrows. In **c** and **d**, spots being specifically altered in RA- or $1,25(OH)_2D_3$-differentiated HL-60 cells in comparison to undifferentiated HL-60 cells are marked with arrow heads. In **c** and **d**, spots being altered in both RA- and $1,25(OH)_2D_3$-differentiated HL-60 cells in comparison to undifferentiated HL-60 cells are marked with arrows. Upward-pointing arrows and arrow heads indicate an increase in protein spot intensity. Down-pointing arrows and arrow heads indicate a decrease in protein spot intensity.

TABLE 1

*Analysis by two-dimensional electrophoresis of alterations in intensity of cytosolic protein spots in HL-60 cells after differentiation with retinoic acid or 1,25-dihydroxy-vitamin $D_3$. Comparison with cytosolic protein spots in human neutrophils.*

| Comparison | Undifferentiated versus differentiated HL-60 cells | | Human neutrophils versus differentiated HL-60 cells | |
|---|---|---|---|---|
| Alterations in intensity of cytostolic proteins | differentiation-inducing agent | | | |
| | retinoic acid | $1,25(OH)_2D_3$ | retinoic acid | $1,25(OH)_2D_3$ |
| All alterations | 64 (10.1%) | 50 (7.9%) | 22 (3.5%) | 25 (4.0%) |
| Protein spots decreased in intensity | 21 (3.3%) | 21 (3.3%) | 11 (1.7%) | 15 (2.3%) |
| Decreases in protein spot intensity common to both differentiations | 13 (2.1%) | | 8 (1.3%) | |
| Protein spots increased in intensity | 43 (6.8%) | 29 (4.6%) | 11 (1.7%) | 10 (1.6%) |
| Increases in protein spot intensity common to both differentiations | 20 (3.2%) | | 7 (1.1%) | |

The intensity of 632 cytosolic protein spots from undifferentiated HL-60 cells was compared with the corresponding intensity of spots from RA- and $1,25(OH)_2D_3$-differentiated HL-60 cells. The alterations in intensity of protein spots in cytosols of differentiated HL-60 cells are referred to the protein pattern of the cytosol of undifferentiated cells (control). In addition, the intensity of those proteins which were found to be altered in intensity in differentiated HL-60 cells was compared with the corresponding protein spots in cytosol of human neutrophils, again undifferentiated HL-60 cells being the reference. The data shown were obtained from the analysis of two different preparations of each cytosol. Triplicate runs with each cytosol preparation were performed. The protein spot patterns were highly reproducible both within repeated runs with one preparation and within different cytosol preparations. Figures indicate the absolute number of proteins altered in intensity. The percentages in parentheses refer to the total number of protein spots studied.

associated with a decrease in amount of 21 proteins (down-pointing arrows and arrow heads in Fig. 1c). In comparison, 43 proteins were present in increased amount in RA-differentiated HL-60 cells in comparison to undifferentiated cells (upward-pointing arrows and arrow heads in Fig. 1c).

In cytosol of $1,25(OH)_2D_3$-differentiated HL-60 cells, 50 proteins were altered in quantity in comparison to undifferentiated cells. Twenty-one of these proteins were present in decreased amount in comparison to undifferentiated cells (down-pointing arrows and arrow heads in Fig. 1d), 13 of them being similarly altered in RA-differentiated HL-60 cells (down-pointing arrows in Figs. 1c and 1d). Twenty-nine proteins were present in increased amount in cytosol of $1,25(OH)_2D_3$-differentiated cells in comparison to undifferentiated HL-60 cells (upward-pointing arrows and arrow heads in Fig. 1d), 20 of these proteins being similarly altered in RA-differentiated cells (upward-pointing arrows in Figs. 1c and 1d).

The differentiation-dependent alterations in protein pattern of differentiated HL-60 cells were compared with the corresponding pattern of cytosolic proteins of

human neutrophils. Twenty-two and 25 of the proteins found to be altered in amount in RA- or $1,25(OH)_2D_3$-differentiated HL-60 cells versus undifferentiated HL-60 cells were similarly altered in human neutrophils in comparison to undifferentiated HL-60 cells (closed and open arrow heads and arrows in Fig. 1b). RA- and $1,25(OH)_2D_3$-differentiated HL-60 cells and neutrophils had 15 of these alterations in common with respect to increases and decreases in protein amount (arrows in Fig. 1b). In addition, RA-differentiated HL-60 cells and neutrophils shared seven increases or decreases in protein amount in comparison to undifferentiated HL-60 cells which had no equivalent in $1,25(OH)_2D_3$-differentiated HL-60 cells (closed arrow heads in Fig. 1b). Conversely, 10 proteins were specifically increased or decreased in amount in $1,25(OH)_2D_3$-differentiated HL-60 cells and neutrophils in comparison to undifferentiated HL-60 cells (open arrow heads in Fig. 1b).

## Discussion

It is generally assumed that culture of HL-60 cells with RA results in neutrophilic differentiation and that culture with $1,25(OH)_2D_3$ leads to monocytic differentiation [4,6,7]. Thus, the four proteins specifically present in increased amount in human neutrophils and RA-differentiated HL-60 cells in comparison to undifferentiated HL-60 cells may be considered as cytosolic marker proteins for neutrophil-like cells (closed arrow heads pointing to the top of the gel in Fig. 1b). However, differentiation of HL-60 cells with RA is also associated with 42 alterations in protein spot intensity which have no equivalent in cytosol of neutrophils. These protein changes may reflect the fact that HL-60 cells are transformed tumour cells which have been cultured in vitro for many years [4–6].

Human neutrophils, $1,25(OH)_2D_3$- and RA-differentiated HL-60 cells share 15 alterations in protein spot intensity (arrows in Fig. 1b). This finding is not surprising as these cells possess a common progenitor cell. Thus, these proteins may be considered as cytosolic marker proteins for both neutrophil-like and monocyte-like cells. Unexpectedly, 10 proteins altered in amount in $1,25(OH)_2D_3$-differentiated HL-60 cells in comparison to undifferentiated HL-60 cells had an equivalent in cytosol of human neutrophils but not in cytosol of RA-differentiated HL-60 cells (open arrow heads in Fig. 1b). These data suggest that differentiation of HL-60 cells with $1,25(OH)_2D_3$ is associated with the partial acquisition of neutrophil-like properties in cytosolic protein pattern and that differentiation with RA does not result in complete acquisition of neutrophil-like properties in cytosolic protein patterns. This assumption is supported by the finding that differentiation of HL-60 cells with $1,25(OH)_2D_3$ but not with RA is associated with the expression of formyl peptide receptors at the plasma membrane in high numbers [7]. In contrast, neutrophilic differentiation of HL-60 cells with dimethyl sulfoxide or dibutyryl cyclic AMP leads to the expression of formyl peptide receptors in high numbers [5,7]. In this context it should be noted that differentiation of HL-60 cells with dibutyryl cyclic AMP and dimethyl sulfoxide is associated with the differential expression of cytosolic components for NADPH oxidase and with differences in the

pattern of cytosolic proteins [2,20]. Other differences between various differentiation procedures resulting in neutrophilic differentiation have been reviewed by Harris and Ralph [6]. Thus, although neutrophilic and monocytic differentiation of HL-60 is clearly defined with respect to morphology, there are dissociations between various neutrophilic differentiation procedures and between neutrophilic and monocytic differentiation at least with respect to cytosolic protein pattern.

A limited number of studies concerning differentiation-dependent changes in protein pattern in RA- and $1,25(OH)_2D_3$-differentiated HL-60 cells has been performed. Murao et al. [8] analysed total cellular proteins of undifferentiated, RA- and $1,25(OH)_2D_3$-differentiated HL-60 cells by 2-DE and radiochemical detection methods. These authors reported that differentiation with RA or $1,25(OH)_2D_3$ is associated with increases in amount of less than 10 proteins. However, the authors did not report on the number of proteins analysed and did not deal in detail with decreases in protein spot intensity. Faille et al. [9] studied alterations in protein phosphorylation pattern and reported that differentiation with RA or $1,25(OH)_2D_3$ is characterized by the appearance of 3–4 phosphoproteins specific for each differentiation pathway. We analysed more than 630 cytosolic proteins by 2-DE and silver-staining and found that 43 and 29 proteins are increased in amount following differentiation with RA or $1,25(OH)_2D_3$ (arrows and arrow heads pointing to the top of the gel in Fig. 1c and d). In addition, the amount of 23 and 9 of these proteins is increased specifically with respect to neutrophilic or monocytic differentiation (arrow heads pointing to the top of the gel in Fig. 1c and d). Thus, we suggest that the 2-DE technique described in this paper is suitable with regard to practicability, sensitivity, resolution and reproducibility to establish specific marker protein patterns for various myeloid cell types.

In conclusion, neutrophilic and monocytic differentiation of HL-60 cells is characterized by a common set of changes in cytosolic protein pattern in comparison to undifferentiated HL-60 cells with respect to increases and decreases. In addition, each differentiation pathway is characterized by specific changes in cytosolic protein pattern. Furthermore, a substantial portion of the proteins found to be altered after neutrophilic and monocytic differentiation possesses equivalents in cytosol of human neutrophils.

## Simplified description of the method

A 2-DE technique is described combining isoelectric focusing with sodium dodecyl sulfate-polyacrylamide electrophoresis and subsequent silver staining. This method is characterized by a high sensitivity, resolution, reproducibility and simple performance. Gel solutions containing all components of the gel with the exception of AP are prepared and stored at $-70°C$, avoiding variability due to lot differences of chemicals. Focused rod gels are stored at $-70°C$, too. By this simplification of the gel preparation and storage, large amounts of gels are produced within a short time. Using this method, differentiation-dependent changes in cytosolic protein patterns in HL-60 leukemic cells were analysed. The changes in protein pattern were compared with the cytosolic protein pattern of human neutrophils. The method described may also be useful for the analysis of differentiation-dependent changes in protein pattern in other cell types.

## Acknowledgements

The authors are grateful to Dr. J. Klose and Dr. G. Schultz for critical discussion, to Mrs. E. Glass and Mrs. A. Schaubitzer for excellent technical assistance and to Dr. E. Keller (Blutbank des Deutschen Roten Kreuzes, Berlin, FRG) for supplying buffy coat preparations.

## References

1 Seifert, R. and Schultz, G. (1987) Reversible activation of NADPH oxidase in membranes of HL-60 human leukemic cells. Biochem. Biophys. Res. Commun. 145, 1296–1302.
2 Seifert, R., Jungblut, P. and Schultz, G. (1989) Differential expression of cytosolic activation factors for NADPH oxidase in HL-60 leukemic cells. Biochem. Biophys. Res. Commun. 161, 1109–1117.
3 Schmidt, H.H.H.W., Seifert, R. and Böhme, E. (1989) Formation and release of nitric oxide from human neutrophils and HL-60 cells induced by a chemotactic peptide, platelet activating factor and leukotriene B4. FEBS Lett. 244, 357–360.
4 Breitman, T.R., Selonick, S.E. and Collins, S.J. (1980) Induction of differentiation of the human promyelocytic leukemia cell line (HL-60) by retinoic acid. Proc. Natl. Acad. Sci. USA 77, 2936–2940.
5 Chaplinski, T.J. and Niedel, J.E. (1982) Cyclic nucleotide-induced maturation of human promyelocytic cells. J. Clin. Invest. 70, 953–964.
6 Harris, P. and Ralph, P. (1985) Human leukemic models of myelomonocytic development: a review of the HL-60 and U937 cell lines. J. Leukocyte Biol. 37, 407–422.
7 Mangelsdorf, D.J., Koeffler, P., Donaldson, C.A., Pike, J.W. and Haussler, M.R. (1984) 1,25-Dihydroxyvitamin $D_3$-induced differentiation in a human promyelocytic leukemia cell line (HL-60): receptor-mediated maturation to macrophage-like cells. J. Cell Biol. 98, 391–398.
8 Murao, S., Gemmell, M.A., Callaham, M.F., Anderson, N.L. and Huberman, E. (1983) Control of macrophage cell differentiation in human promyelocytic HL-60 leukemia cells by 1,25-dihydroxyvitamin $D_3$ and phorbol 12-myristate 13-acetate. Cancer Res. 43, 4989–4996.
9 Faille, A., Poirier, O., Turmel, P., Chomienne, C., Charron, D.J. and Abita, J.P. (1986) Changes in the patterns of protein phosphorylation associated with granulocytic and monocytic-induced differentiation of HL-60 cells. Anticancer Res. 6, 1053–1063.
10 Bernal, S.D. and Chen, L.B. (1982) Induction of cytoskeleton-associated proteins during differentiation of human myeloid leukemic cell lines. Cancer Res. 42, 5106–5116.
11 Seifert, R. and Schultz, G. (1987) Fatty acid-induced activation of NADPH oxidase in plasma membranes of human neutrophils depends on neutrophil cytosol and is potentiated by stable guanine nucleotides. Eur. J. Biochem. 162, 563–569.
12 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.
13 Klose, J. (1983) High resolution of complex protein solutions by two-dimensional electrophoresis. In: Tschesche, H. (Ed.), Modern Methods in Protein Chemistry-Review Articles, Walter de Gruyter, Berlin, pp. 49–78.
14 O'Farrell P.Z., Goodman H.M. and O'Farrell P.H. (1977) High-resolution two-dimensional electrophoresis of basic as well as acidic proteins. Cell 12, 1133–1142.
15 Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.
16 Merril, C.R., Dunau, M.L. and Goldman, D. (1981) A rapid silver stain for polypeptides in polyacrylamide gels. Anal. Biochem. 110, 201–207.
17 Heukeshoven, J. and Dernick, R. (1985) Simplified method for silver staining of proteins in polyacrylamide gels and the mechanism of silver staining. Electrophoresis 6, 103–112.

18 Jungblut, P. and Klose, J. (1986) Composition and genetic variability of Heparin-Sepharose CL-6B protein fractions obtained from the solubilized proteins of mouse organs. Biochem. Genet. 24, 925–939.

19 Jungblut, P., Schneider, W. and Klose, J. (1985) Quantitative analysis of two-dimensional electrophoretic protein patterns: Comparison of visual evaluation with computer-assisted evaluation. In: Neuhoff, V. (Ed.), Electrophoresis'84, Verlag Chemie, Weinheim, pp. 301–303.

20 Jungblut, P. and Seifert, R. (1988) Differential expression of cytosolic activation factors for NADPH oxidase in HL-60 cells. Biol. Chem. Hoppe-Seyler 369, 845.

21 Gianazza, E., Astrua-Testori, S., Caccia, P., Giacon, P., Quaglia, L. and Righetti, P.G. (1986) On the reproducibility of spot position in electrophoretic separations. Electrophoresis 7, 76–83.