Proc. Natl. Acad. Sci. USA
Vol. 76, No. 6, pp. 2779–2783, June 1979
Cell Biology

Human promyelocytic leukemia cells in culture differentiate into macrophage-like cells when treated with a phorbol diester

(12-*O*-tetradecanoylphorbol 13-acetate/adhesion/terminal differentiation/myeloid differentiation/monocytes)

GIOVANNI ROVERA, DANIELA SANTOLI, AND CAROLINE DAMSKY

The Wistar Institute of Anatomy and Biology, 36th Street at Spruce, Philadelphia, Pennsylvania 19104

Communicated by Hilary Koprowski, February 15, 1979

ABSTRACT **When suspension cultures of human promyelocytic leukemia cells (line HL60) were treated with 12-*O*-tetradecanoylphorbol 13-acetate (TPA; 1.6–160 nM), more than 80% of the cells adhered to the plastic substrate within 24 hr. Within the same time period the immature azurophilic granulations typical of HL60 promyelocytic cells disappeared and the nuclear chromatin became more condensed, but the nucleolus was retained. The attached cells stopped dividing and synthesizing DNA. The phenomenon was irreversible and independent of the continuous presence of TPA. Approximately 60% of the untreated cells and of TPA-treated cells bore surface Fc receptors for IgG. Under the experimental conditions used, about 10% of the TPA-treated cells were also able to phagocytize IgG-coated erythrocytes and more than 80% were able to phagocytize latex beads, but untreated controls were unable to do so. Cellular levels of NADase, acid phosphatase, and nonspecific esterase were markedly increased after treatment with TPA, whereas little or no increase was seen after treatment with dimethyl sulfoxide (Me_2SO), a drug that induces myeloid differentiation of HL60 cells. Peroxidase activity was lower in TPA-treated and Me_2SO-treated cells than in HL60 cells. More lysozyme was found in the medium of TPA-treated cells than in the medium of untreated or Me_2SO-treated cells. These data indicate that, after treatment with TPA, human promyelocytic leukemia cells can differentiate into cells that have several characteristics of macrophages.**

The human promyelocytic leukemia line HL60 established by Collins *et al.* (1) consists predominantly of promyelocytes (85%) and of a small fraction of more mature myeloid elements that can be efficiently increased by treatment with dimethylsulfoxide (Me_2SO), butyric acid, or dimethylformamide (2).

It has been reported that some phorbol diesters, of which 12-*O*-tetradecanoylphorbol 13-acetate (TPA) is the most active, interfere in culture with the process of spontaneous or induced differentiation of Friend erythroleukemia cells and of several other cell systems (3–9). It has also been reported that in some lines of murine erythroleukemic cells TPA induces rather than inhibits differentiation (10). We report now that TPA treatment changes HL60 cells into terminally differentiated cells with several characteristics of macrophages.

MATERIALS AND METHODS

Cells. HL60 human promyelocytic leukemic cells were grown as described (2) with 15% fetal calf serum. TPA (Midland Corp.) solubilized in acetone at 0.16 mM was added to the cell suspension at the final concentrations detailed in *Results*. Control cultures contained equivalent amounts of acetone. For morphological examination, 0.1 ml of cell suspension was centrifuged onto slides by using a Shandon–Elliot Cytospin centrifuge. Cells were stained with May–Grünwald–Giemsa.

Monocytes and granulocytes from healthy donors prepared as described (11) and human fetal skin fibroblasts FS2 (11) were used in some experiments.

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Table 1. Adherence of HL60 cells after treatment with TPA

Treatment time, hr	TPA, nM	Number of cells	
		Suspended	Adherent
0–⅓	160	$<10^4$	1.4×10^6
	16	4.6×10^6	$<10^4$
0–3	16	1.1×10^6	3.5×10^5
0–24	16	3.0×10^5	1.1×10^6
0–48	16	1.0×10^5	1.2×10^6
	0.16	4.8×10^6	$<10^4$

Cells (1.5×10^6) were treated with TPA at the concentration and for the length of time indicated. The numbers of suspended and adherent cells were determined at 48 hr and after the removal of TPA.

Transmission Electron Microscopy. Untreated and TPA-treated cells were fixed in 4% (wt/vol) glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, for 30 min at 4°C (12) followed by 1% OsO_4 in the same buffer for 30 min at 4°C. Preparations were stained with saturated aqueous uranyl acetate (2 hr at 4°C), dehydrated in ethanol, and embedded in araldite/Epon. Sections were cut perpendicular to the substrate and examined in a Zeiss 10 electron microscope.

Presence of Fc Receptors for IgG or IgM. Cells were examined for the presence of Fc-IgG or Fc-IgM receptors by testing their ability to form rosettes with ox erythrocytes (OE) sensitized with purified rabbit IgG (IgG-OE) or IgM (IgM-OE) antibodies (13, 14).

Phagocytic Activity. Uptake of latex beads was determined as described by Levine and Cox (15). For the erythrophagocytosis studies, 1 ml of a 0.5% suspension of IgG-OE, IgM-OE, or unsensitized OE was added to 5 ml of culture medium with or without serum for 3–24 hr. The monolayers were washed repeatedly, and after trypsinization, cytocentrifuge slides were prepared and stained with benzidine.

Enzymatic Assays. Acid phosphatase (EC 3.1.3.2) was determined as described by Schnyder and Baggiolini (16), and NADase (EC 3.2.2.5) as described by Artman and Seeley (17). The amount of lysozyme (EC 3.2.1.17) present in the medium was calculated as described by Litwack (18). Peroxidase activity (EC 1.11.1.7) was determined by using *o*-dianisidine (19). Acid α-napthylacetate esterase (EC 3.1.1.2) activity was determined as described by Mueller *et al.* (20).

Protein Synthesis. HL60 cells were labeled for 24 hr with a ^{14}C-labeled amino acid mixture (1 μCi/ml) (1 Ci = 3.7×10^{10} becquerels). After repeated washing of the cultures with phosphate-buffered saline, the cells were resuspended and counted. The amount of radioactive precursor incorporated was determined in aliquots after 10% trichloroacetic acid precipitation on glass filters.

DNA Synthesis. DNA synthesis was evaluated by determining the extent of [^3H]thymidine (specific activity 6.7 Ci/

Abbreviations: TPA, 12-*O*-tetradecanoylphorbol 13-acetate; Me_2SO, dimethylsulfoxide; OE, ox erythrocyte.

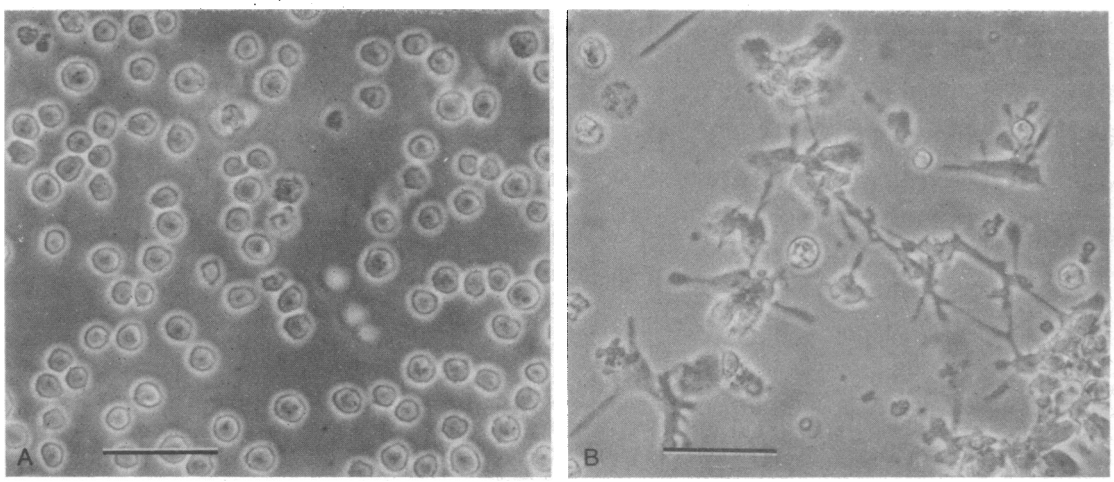

FIG. 1. Morphological changes after TPA treatment of HL60 human promyelocytic leukemia cells. (*A*) Not treated; (*B*) TPA-treated for 24 hr. (Phase contrast; bar represents 100 μm.)

mmol) incorporated during 24-hr labeling pulses into trichloroacetic acid-insoluble fractions or by autoradiography (21).

RESULTS

Changes in Adherence after Treatment with TPA. Whereas untreated HL60 cells grew as single cell suspension cultures (Fig. 1*A*), as soon as 1 hr after exposure to TPA (1.6–160 nM), few cells were adherent. After 24 hr, 80% of the cells were attached to the substrate, very often in small clumps (Fig. 1*B*). The cells could not be detached by vigorous shaking but could be detached by treatment with trypsin/EDTA for 10 min.

After 48 hr of continuous treatment with TPA, more than 95% of the cells were adherent. The cells that remained in suspension after this time were not viable. At this stage adherent cells could be removed from the plastic substrate only by prolonged (about 30 min) trypsin treatment or scraping. Five to 6 days after TPA treatment, the cells began to detach.

To test the reversibility of the TPA-induced changes, 1.5×10^6 cells were treated with various doses of TPA for various lengths of time. The drug was then removed, by repeated washing of the cultures, and the numbers of adherent and nonadherent cells were determined after 48 hr (Table 1). When cells were treated with 160 nM TPA for as short a time as 20 min, more than 90% of the cells were adherent 48 hr later and unable to proliferate further despite the removal of the drug. The small fraction of nonattached cells was not viable. A lower concentration of TPA (16 nM) required more than 3 hr of contact with the cells in order to produce the adhesive response and a concentration of 0.16 nM was totally ineffective. Treatment of HL60 cells with Me_2SO for 5 days produced only a small number (less than 0.1%) of adherent cells.

Morphological Changes Induced after TPA Treatment. Untreated HL60 cells stained with May–Grünwald–Giemsa showed extensive cytoplasmic azurophilic granulation masking the nucleus. One or several nucleoli were present (Fig. 2*A*). After 24 hr of treatment with TPA, most of the azurophilic granules disappeared (Fig. 2*B*), but the cytoplasm remained basophilic. The nucleus was reniform or irregular with one or two nucleoli. After 48 hr, the cytoplasm became less basophilic and large droplets (staining positively for oil red 0) became increasingly evident (Fig. 2*C*).

With transmission electron microscopy, the most distinctive feature of the HL60 cells was the presence of large cytoplasmic vacuoles that contained loosely packed floccular material (Figs. 3 *A* and *B*). Smaller cores of tightly packed material were present within some vacuoles. These dense-cored vesicles most likely correspond to the azurophilic granules observed in Fig. 2*A*.

After a 72-hr exposure to TPA, adherent cells were flattened and showed a wide variety of shapes with long, thin processes extending from the margins of many cells (Fig. 3*C*). The nucleolus remained prominent, in contrast to its absence in Me_2SO-induced HL60 and in myeloid cells past the promyelocytic stage (Fig. 3*C*). The dense-cored vacuoles present in suspension cells were absent and smaller vacuoles with uniformly dense cores were present, localized along with small coated vesicles, in the vicinity of the extensive Golgi apparatus (Fig. 3*D*). Lipid droplets were abundant in TPA-treated cells, as were mitochondria and rough endoplasmic reticulum.

Patterns of Cell Multiplication and DNA and Protein Synthesis after TPA Treatment. The cell number and the amount of [^{3}H]thymidine and ^{14}C-labeled amino acids incorporated into acid-insoluble fractions during 24-hr periods were determined in HL60 cells treated for different lengths of time with TPA (Fig. 4). Associated with arrest in cell proliferation was a sharp decrease in the amount of [^{3}H]thymidine incorporated into DNA. Autoradiographic analysis, using 24-hr pulse labeling, indicated that more than 90% of the untreated HL60

Table 2. Enzymatic activities in treated and untreated HL60 cells

Treatment	Lysozyme,* μg	Enzyme units† per 5×10^6 cells: NADase	Acid phosphatase	Myeloperoxidase	Nonspecific esterase‡
None	1.1	0.3	0.0025	0.14	±
TPA (16 nM, 4 days)	16.5	3.4	0.0420	0.01	+++
Me_2SO (1.2% by wt, 5 days)	3.0	0.3	0.0057	0.02	±

All values represent averages of at least three separate experiments.

* Amount of enzyme released into the medium by 5×10^6 cells in a 96-hr period; medium alone showed no detectable activity.

† Enzyme units: NADase, amount of enzyme that cleaves 1 μmol of NAD in 5 min at 37°C; acid phosphatase, amount of enzyme that hydrolyzes 1 μmol of *p*-nitrophenylphosphate per min at 26°C; myeloperoxidase, amount of enzyme decomposing 1 μmol of peroxide per min at 25°C.

‡ Intensity of brown color developed histochemically.

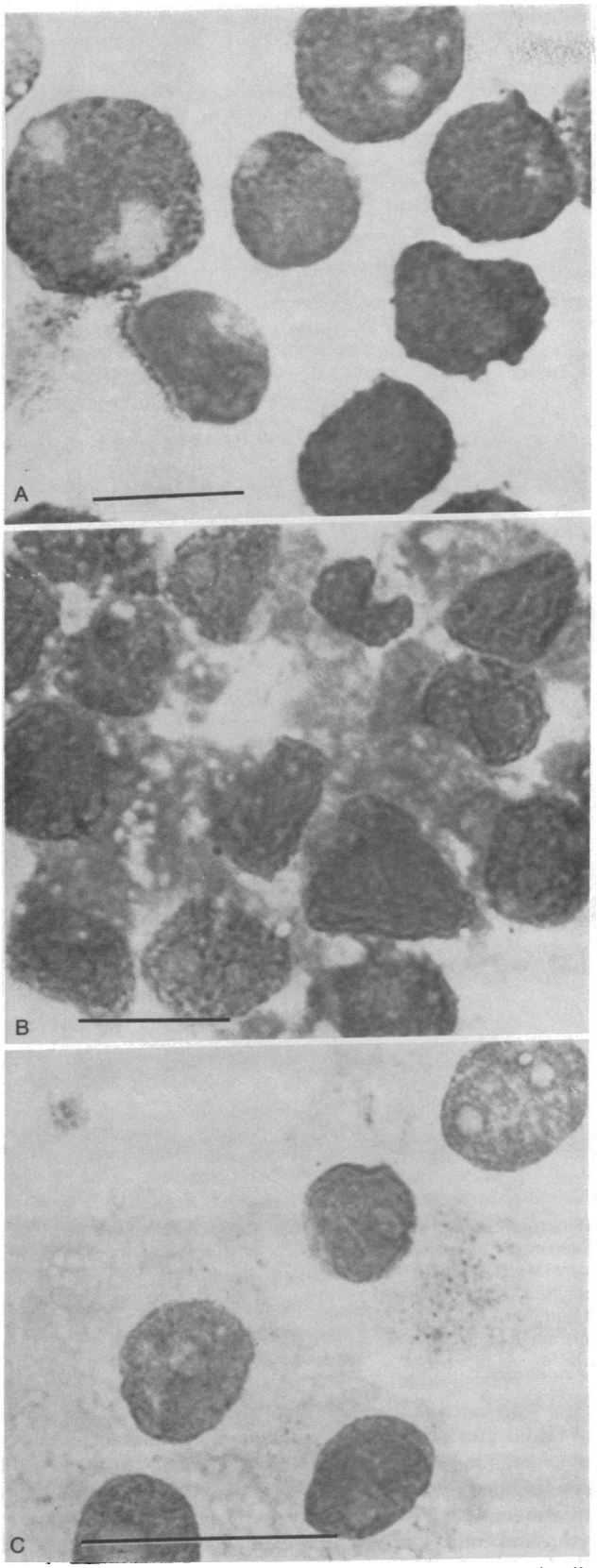

FIG. 2. Light microscopy of HL60 cells. (*A*) Untreated cells; (*B*) cells treated with TPA for 24 hr; (*C*) cells treated with TPA for 4 days. (May-Grünwald-Giemsa; bar represents 20 μm; ×950.)

cells were synthesizing DNA in a 24-hr period, whereas after 24 hr of TPA treatment only 12% of the cells and after 48 hr essentially none synthesized DNA (data not shown). After a transient increase in the first 24 hr, the amount of amino acids incorporated into protein in TPA-treated cells was also reduced by approximately 50%. Taken together, these data indicate that after TPA treatment HL60 promyelocytes rapidly lose proliferative capacity but are still able to synthesize proteins.

Surface Markers Characterization. Untreated and TPA-treated HL60 cells did not form rosettes with unsensitized OE or IgM-OE but formed rosettes with IgG-OE, indicating the presence of Fc receptors for IgG. The number of rosetting cells increased with incubation at 4°C (dati not shown). A maximum of 65% rosetting cells was observed in both untreated and TPA-treated HL60 cells after overnight incubation. Freshly isolated trypsinized human monocytes were 75–98% positive, and the peak of rosetting cells was reached after only 20 min of incubation. Six-day-old human monocytes had the same percentage of IgG-rosetting cells as did HL60 cells. Human FS2 fibroblasts did not form rosettes.

Phagocytic Activity of TPA-Treated Cells. The phagocytic activity of untreated and TPA-treated HL60 cells was tested by incubating cells in the presence of 1-μm latex beads or in the presence of OE. Untreated cells were not able to incorporate latex beads. The rate of incorporation increased as a function of length of exposure to TPA, reaching a peak 4 days after treatment. Human fibroblasts exhibited an even better ability to phagocytize latex beads. In the absence of serum, a fraction of TPA-treated cells (approximately 10%) were able to phagocytize IgG-OE but not IgM-OE or unsensitized OE. Erythrophagocytosis was more efficient in human monocytes (65% were able to phagocytize IgG-OE but less than 0.5%, IgM-OE). Human fibroblasts were not capable of phagocytizing either sensitized or unsensitized OE. In the absence of serum, fresh human granulocytes showed erythrophagocytosis only with IgG-OE, but the level of erythrophagocytosis was low (less than 0.1%). Erythrophagocytosis was not seen in Me_2SO-treated HL60 cells in either the presence or the absence of serum. Presence of serum in the incubation medium reduced erythrophagocytosis ability of TPA-treated cells to approximately 1%.

Comparison of Enzymatic Activities of Me_2SO- and TPA-treated HL60. A number of enzymatic activities were compared in HL60 cells induced to differentiate by Me_2SO along the myeloid lineage or after TPA treatment. The data are summarized in Table 2. NADase, an enzyme recently reported to be a characteristic marker of monocytes and macrophages (17), increased more than 10-fold after TPA treatment and did not increase after Me_2SO treatment. Acid phosphatase and nonspecific acid esterase (two other enzymes typical of macrophages) also increased severalfold after TPA treatment and little, or not at all, after Me_2SO treatment. On the contrary, myeloperoxidase activity was markedly decreased in TPA-treated cells as expected for monocytes and macrophages. Lysozyme activity was present in the medium of control HL60 cells and increased after treatment with TPA but very little with Me_2SO. Neither the supernatant of human fibroblasts nor the medium incubated alone displayed detectable activity.

DISCUSSION

Tissue macrophages are derived from blood monocytes (22), which, in turn, originate in bone marrow (23). Cloning of bone marrow stem cells *in vitro* has indicated that a common stem cell is shared by myeloid and monocytic elements (24, 25). The earliest recognizable form of the mononuclear phagocyte series is the promonocyte (23). When explanted on plastic or glass surfaces, promonocytes differentiate into macrophages (22).

Mixed cultures of granulocytes and macrophages are obtained from normal or leukemic myeloid cells in culture that are induced to differentiate by various inducers or serum factors (26), but in at least one case leukemic mouse myeloid cells could

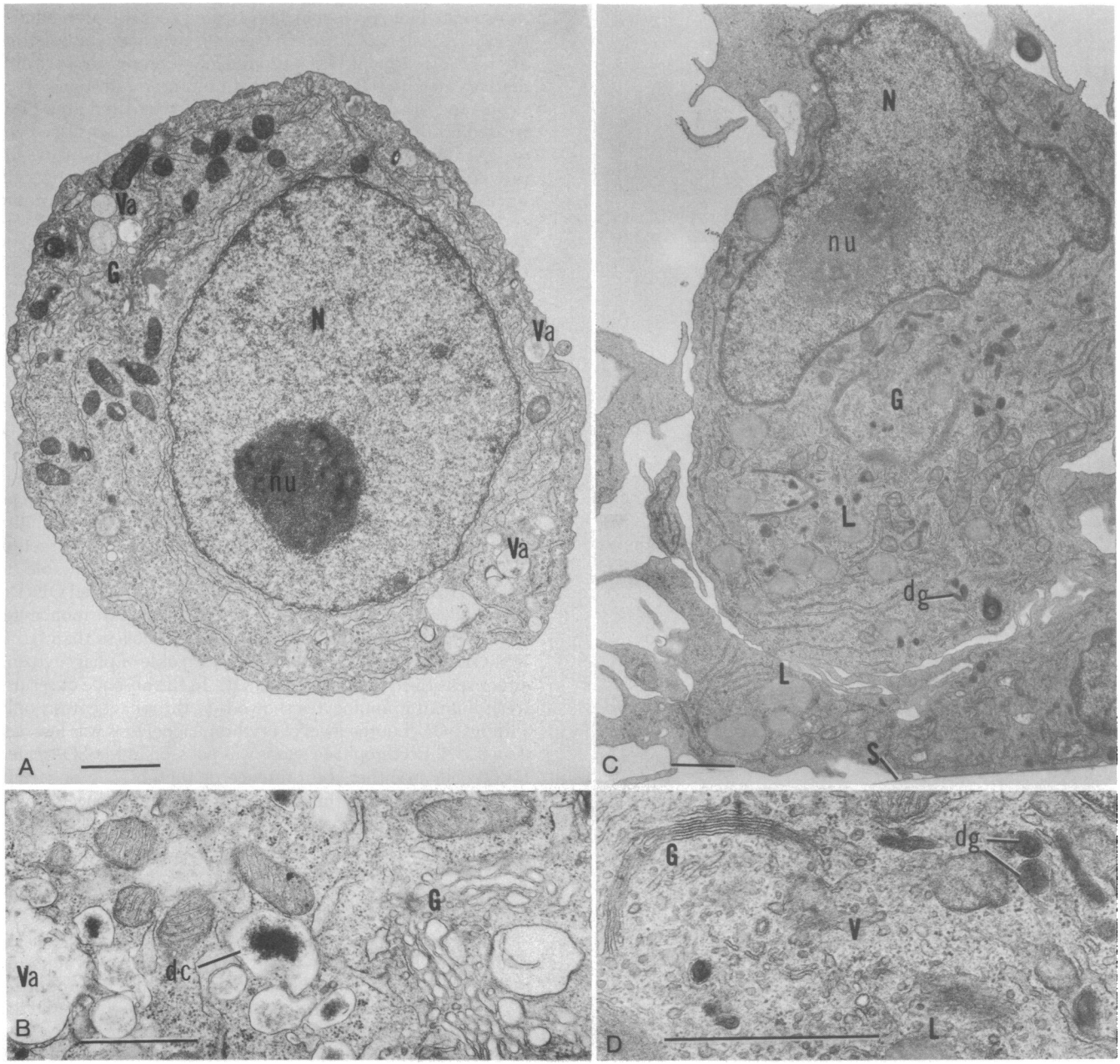

FIG. 3. (*A, B*) HL60 cells. Cells contain a large nucleus (N) with diffuse chromatin, a prominent nucleolus (nu), and a well-defined Golgi apparatus (G) associated with a variable number of large vacuoles (Va). The content of the vacuoles is flocculent and loosely packed in most cases (*A*), but many vacuoles contain a core (dc) of more densely packed material (*B*). (*C*) HL60 cells treated with 16 nM TPA for 72 hr. Long surface extensions are evident. The nucleus is reniform with a distinct nucleolus. Lipid droplets (L) and small uniformly dense granules (dg) are apparent. The number of mitochondrial profiles and the extent of rough endoplasmic reticulum increased in TPA-treated cells. (*D*) Higher magnification of the Golgi region of the TPA-treated cell in *C*. A large number of small vesicles (V) are present in close proximity to Golgi cisternae. Dense granules are also localized in the Golgi region. (Bar represents 1 μm.)

be specifically induced to differentiate into cells that, purely on the basis of adhesiveness to substrate and presence of IgG receptors, were identified as macrophages (27).

In this paper we present evidence that a phorbol diester (TPA) can induce differentiation of an established line of human promyelocytic leukemia cells (HL60) into a cell type with several of the characteristics of macrophages. This is in contrast to treatment of these same cells with Me_2SO, which results in their differentiation into cells with several characteristics of granulocytes (2).

Monocytes and TPA-treated HL60 cells adhere strongly to the substrate and can be detached by trypsinization in the first 24 hr, but in both cases after 48 hr the cells become relatively resistant to trypsin treatment (22). Adhesion to a substrate is poor both in myeloid elements, obtained by Me_2SO treatment of HL60, and in human granulocytes in the presence of medium containing serum. Fibroblasts adhere well but can be easily resuspended by trypsinization. Increased adhesion caused by treatment with TPA has been reported in a clone of mouse erythroleukemic cells (28) and in chicken chondroblasts (8). In the former case, however, the cells continued to proliferate, and in the latter case even the nontreated culture eventually became adherent. The ability to phagocytize latex beads is shown by fibroblasts (29), neutrophilic granulocytes (30), monocytes (31), and TPA-treated HL60 cells. Phagocytosis has also been reported in nonadherent Me_2SO-treated HL60 (2) and is, therefore, not necessarily dependent on the phenomenon of cell adhesion. However, under our experimental conditions, the

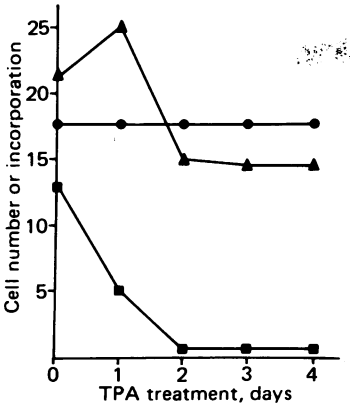

FIG. 4. Cell number and incorporation of ^{14}C-labeled amino acids and [^{3}H]thymidine into cells treated for different lengths of time with TPA. ●, Cell number (cells × 10^{-5} per ml); ▲, ^{14}C-labeled amino acid incorporation into acid-insoluble fractions (cpm × 10^{-3} per cell); ■, [^{3}H]thymidine incorporation into acid-insoluble fractions (cpm × 10^{-3} per cell).

ability to specifically ingest IgG-coated erythrocytes is seen only in monocytes and TPA-treated cells and not in Me_2SO-treated HL60 or in normal fibroblasts and is minimal or absent in mature granulocytes. The presence of surface receptors for the Fc fragment of IgG has been reported in monocytes (32) but also at an early phase of neutrophil differentiation (30). Fc-IgG receptors are not present in normal fibroblasts.

The ultrastructure of untreated HL60 leukemic promyelocytes resembles that of early promyelocytes in: size and shape of nucleus; structure of chromatin and presence of nucleolus; presence of well-developed Golgi apparatus and large, immature, dense-cored azurophilic granules; and structure and extent of rough endoplasmic reticulum and mitochondria (33).

After TPA treatment, these cells resemble cells of the monocyte/macrophage line in many respects and do not resemble end cells of the myeloid series (metamyelocytes or granulocytes). The nuclear morphology of terminally differentiated cells of myeloid series (from metamyelocyte) is segmented and nucleoli are absent in myeloid cells that have passed the myelocytic stage. On the contrary, TPA-treated cells and monocyte/macrophage cells have a round or reniform nucleus and prominent nucleolus. Ultrastructural features that TPA-treated cells and monocyte/macrophage cells have in common include prominent Golgi associated with small coated vesicles and small granules with uniform density, extensive rough endoplasmic reticulum, and numerous mitochondria. The resemblance is not complete, however, because mature stimulated macrophages contain large digestive vacuoles (secondary lysosomes) that are not found in TPA-treated cells.

During the differentiation process of TPA-treated HL60 cells, there is no phase in which mature monocytes can be recognized. However, 24 hr after TPA treatment, cells without azurophilic granulations and with an indented nucleus can be seen. This observation is similar to that of van Furth and Cohn (23), who described a direct transition from promonocyte to macrophage in culture. It is possible that development of a well-differentiated monocyte requires the presence of an environment similar to the intravascular environment, which does not allow adhesion and spreading of the cells. The possibility of directing in culture cellular differentiation of HL60 cells toward either the myeloid (2) or the monocytic macrophage-series (our data) will be helpful in elucidating the interactions between cells of these two closely related lineages.

We are grateful to Dr. S. J. Collins and Dr. R. C. Gallo for the gift of human promyelocytic leukemia (HL60) cells. The technical assistance of Elsa Aglow, Sena Smith, and Pacifico Meo is greatly appreciated. This research was supported by U.S. Public Health Service Research Grants CA-21124, CA-21319 and CA-23413 from the National Cancer Institute, RR-05540 from the Division of Research Resources, IM-168 from the American Cancer Society, and a grant from the National Multiple Sclerosis Society. G.R. is a scholar of the Leukemia Society of America.

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