**DIFFERENTIATION OF HUMAN LEUKEMIAS IN RESPONSE TO 12-O-TETRADECANOYLPHORBOL-13-ACETATE IN VITRO**

By Luigi Pegoraro, Janet Abraham, Richard A. Cooper, Alessandro Levis, Beverly Lange, Pacifico Meo, and Giovanni Rovera

Leukemic cells from patients with acute myeloid leukemia underwent morphological, functional, and histochemical changes within 24–48 hr after treatment with 1.6 x 10^-10 M 12-O-tetradecanoylphorbol-13-acetate (TPA). The changes included adhesion to the plastic substrate, a 4–6-fold increase in the number of phagocytic cells, and an increase in the number of alpha-naphthyl-acetate esterase (α-NAE) positive cells. In contrast, TPA treatment of cells from patients with acute lymphoblastic leukemia caused some aggregation of cells in suspension, but no changes in adhesion, phagocytosis, or α-NAE. Of the four cases of undifferentiated or unclassified leukemias studied, two failed to respond to TPA, one responded with a myeloid (adhesion) pattern, and one with a lymphoid (aggregation) pattern. These data suggest that leukemic myeloblasts retain the ability to express a variety of differentiated functions, and in some cases, it may be possible to use TPA as a tool to test the differentiative potential of undifferentiated human leukemias.

The most efficient phorbol diester in the modulation of differentiation of established leukemic cell lines is 12-O-tetradecanoylphorbol-13-acetate (TPA). At doses ranging from 10^-5 to 10^-10 M, TPA inhibits erythroid differentiation of Friend erythroleukemia cells and myeloid differentiation of M1 mouse myeloid leukemia cells, and it stimulates differentiation of cloned cell lines of Rauscher virus-induced mouse erythroleukemia.

TPA has also been found to induce differentiation of the human promyelocytic leukemia cell line, HL-60, into macrophages. Similar results have been observed with certain clones of mouse myeloid leukemia. Macrophage differentiation has been characterized by the appearance of two acid phosphatase isoenzymes and of α-NAE, marker enzymes present in monocytic but not in myeloid cells, and by decreased activity of peroxidase and chloroacetate esterase (Rovera, unpublished), two enzymes abundant in myeloid cells but with low or absent activity in monocytes. In addition, a series of changes has been induced with TPA that are characteristic of both mature granulocytes and monocytes/macrophages which, although not useful in defining a specific direction of differentiation, are indicative of the complex program of differentiation that TPA induces in myeloid leukemia cell lines. These include an increase in lysozyme activity, the arrest of cell proliferation, adhesion to plastic surfaces, phagocytosis and immunoenzyme/haptoaggregation, and an increase in the number of C3 receptors.

We questioned whether these effects of TPA were limited to leukemia cell lines in culture or whether they represented a more general response of leukemic cells in patients. Therefore, we examined the effects of TPA on cells obtained directly from the peripheral blood or bone marrow of patients with acute leukemia.

**MATERIALS AND METHODS**

**Patient Population**

Twenty-nine leukemic patients were studied, 23 of whom were studied at the time of diagnosis prior to any therapy, 3 at the time of relapse only, and 3 at diagnosis and subsequent relapse. Leukemias were diagnosed based on cell morphology and cytogenetic staining and classified according to the French-American-British scheme. The distribution of patients according to the morphological type of leukemia is shown in Table 1.

**Leukocyte Preparation**

Heparinized peripheral blood (20 ml) or bone marrow (1 ml) was obtained during routine diagnostic procedures. Only specimens containing at least 70% leukemic blasts were studied. Cells were diluted with RPMI-1640 medium, supplemented with 15% fetal bovine serum overlayed at 20°C on a cushion of 5 ml Ficoll-Hypaque, centrifugated at 1000 g for 30 min. The leukemic immature marrow cells and mononuclear cells present in the interface were washed once, plated on Falcon flasks at 1–5 x 10^6 cells/ml, and incubated at 37°C with 5% CO2 and humidity. TPA (from P. Borchert, Eden Prairie, Minn.) in acetone was added to the...
cultures at the time of plating at a final concentration of $1.6 \times 10^8$ M. Control cultures received the equivalent amount of acetone (0.1%).

**Adhesion and Aggregation**

After 24 hr in culture, adherence of cells was scored by counting the number of cells adhering to the plastic flask. Aggregation was evaluated by counting the number of cells forming clumps in suspension.

**Histochemistry**

After 72 hr in culture, cells were collected on a glass slide with an Elliott-Shandon cytocentrifuge. Naphthol ASD chloroacetate esterase and $\alpha$-NAE activities were determined histochemically as described. Only cells staining intensely brown-red were considered positive for $\alpha$-NAE activity. Sudan Black, peroxidase, and periodic acid-Schiff (PAS) staining were done as described.

**Phagocytosis**

After 24 hr, a 10-μl volume of polystyrene latex beads (Poly-sciences Inc., 0.93 μm in diameter) was added to the cultures and left in contact with the cells for 48 hr. In some cases, Saccharomyces cerevisiae, pretreated with fresh human serum, were incubated with the cells 3 days after the beginning of the cultures for 30 min. Cells containing at least 5 latex beads or one Saccharomyces cerevisiae per cell were considered phagocytes.

### Results

#### Adhesion and Aggregation

The ability of TPA, when added in vitro to different types of human leukemic cells, to induce adhesion to a plastic substrate is summarized in Table 1. Treatment of human lymphoblastic leukemia cells with TPA did not produce detectable changes in 6 of 9 of the cases. In the other 3 cases, treatment with TPA induced the aggregation of 5–20 cells, a small percentage of which adhered to the plastic surface and were detachable by shaking. In all cases of acute myeloid leukemia, TPA treatment induced adhesion in a large fraction of the cell population. Seventy-seven percent ($\pm 4.8\%$) of leukemic myeloblasts became adherent 24 hr after TPA treatment, in contrast to 21.2% ($\pm 12.4\%$) in control cultures. The adherent cells were not detachable by shaking but could be detached by trypsinization for 15–30 min. In one case of myelomonocytic leukemia, and in one of two cases of promyelocytic leukemia, TPA treatment induced adhesion to an extent comparable to that observed in acute myeloblastic leukemia. In three cases of monocytic leukemia, adhesion was observed in untreated cells and was not increased by TPA treatment.

No response was obtained after TPA treatment in two of four cases of undifferentiated leukemia. In the other two cases, either adhesion or massive clumping associated with adhesion was observed. The latter case had originally been classified in one institution as myeloblastic and in another institution as lymphoblastic.

#### Phagocytosis

The percentage of phagocytic cells in untreated and TPA-treated leukemic cells is given in Table 2. Phagocytic activity was tested in about half of the cases examined for adhesion in response to TPA treatment: the number of cells with phagocytic activity was found to increase in all the cases in which adhesion increased. Lymphoblastic leukemias showed very low levels of phagocytosis, possibly due to contamination with other mononuclear cells. Phagocytosis was present in monocytic or myelomonocytic leukemias, in agreement with the report of Neuwirtova et al. After TPA treatment, it increased in both disorders, but more in the myelomonocytic leukemia. The number of phagocytic cells was very low in the two cases of undifferentiated leukemia cells, which showed massive clumping and adhesion (see previous section).

### Table 1. Adhesion and Aggregation of Leukemic Cells

<table>
<thead>
<tr>
<th>Type of Leukemia</th>
<th>Number of Cases</th>
<th>Adhesion†</th>
<th>TPA-Treated*</th>
<th>Aggregation‡</th>
<th>TPA-Treated*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myeloblastic</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Promyelocytic</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Myelomonocytic</td>
<td>3</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Monocytic</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Lymphoblastic</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Undifferentiated</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*TPA ($1.6 \times 10^{-8}$ M) was added to the cultures at the beginning of the experiment.

†Adhesion (number of cells that adhered to the plastic surface and could not be removed by shaking) and aggregation (number of cells that formed clumps of more than 10 cells) were determined after 24 hr. Number of cases showing adhesion (a value of up to 25% adherent cells was considered negative). See text for mean values and standard deviation.

‡Number of cases with aggregates of more than 10 cells.

§Highly undifferentiated or of questionable classification.

### Table 2. Percent of Phagocytic Cells After TPA Treatment

<table>
<thead>
<tr>
<th>Type of Leukemia</th>
<th>Number of Cases Tested</th>
<th>Untreated†</th>
<th>TPA-Treated†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myeloblastic</td>
<td>5</td>
<td>16.0 (± 7.1)</td>
<td>57.2 (± 2.2)</td>
</tr>
<tr>
<td>Promyelocytic</td>
<td>1</td>
<td>5.0</td>
<td>30.0</td>
</tr>
<tr>
<td>Myelomonocytic</td>
<td>3</td>
<td>15.6 (± 4.8)</td>
<td>51.0 (± 12.3)</td>
</tr>
<tr>
<td>Monocytic</td>
<td>2</td>
<td>38.5</td>
<td>42.1</td>
</tr>
<tr>
<td>Lymphoblastic</td>
<td>8</td>
<td>4.1 (± 4.0)</td>
<td>4.6 (± 3.2)</td>
</tr>
<tr>
<td>Undifferentiated</td>
<td>3</td>
<td>5.5 (± 4.1)</td>
<td>2.5 (± 1.5)</td>
</tr>
</tbody>
</table>

*TPA ($1.6 \times 10^{-8}$ M) was added at the beginning of the experiment.

†Mean and standard deviations.
DIFFERENTIATION OF HUMAN LEUKEMIAS

Table 3. Esterase Activity

<table>
<thead>
<tr>
<th>Type of Leukemia</th>
<th>Number of Cases Tested</th>
<th>α-NAE*</th>
<th>N-ASDCA†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Whole Bone Marrow or Peripheral Blood</td>
<td>Untreated</td>
<td>TPA-Treated</td>
</tr>
<tr>
<td>Myeloblastic</td>
<td>5</td>
<td>3 (±2)</td>
<td>6.8 (±3.1)</td>
</tr>
<tr>
<td>Promyelocytic</td>
<td>1</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>Myelomonocytic</td>
<td>3</td>
<td>28 (±17)</td>
<td>24 (±14)</td>
</tr>
<tr>
<td>Monocytic</td>
<td>3</td>
<td>77 (±22)</td>
<td>83 (±6)</td>
</tr>
<tr>
<td>Lymphoblastic</td>
<td>6</td>
<td>0.4 (±0.5)</td>
<td>0.5 (±0.4)</td>
</tr>
<tr>
<td>Undifferentiated</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Cells were kept in culture for 3 days with or without TPA prior to the histochemical assay.
*Percent of α-NAE-positive cells (mean and standard deviation).
†Percent of naphthol ASD chloracetate esterase-positive cells (mean and standard deviation).

Esterase Activity

In all cases of acute myeloblastic leukemia and myelomonocytic leukemia tested, the number of cells showing α-NAE activity (a marker of monocytic but not of myeloid elements) increased within 3 days after treatment with $1.6 \times 10^{-4} M$ TPA. α-NAE activity was high in untreated monocytic leukemia and remained unchanged after TPA treatment. The percentage of cells containing naphthol ASD chloracetate esterase (a myeloid marker) always decreased after TPA treatment. Table 3 shows the data on esterase activity and compares the enzymatic activities of TPA-treated bone marrow of peripheral blood prior to separation with the blast-cell-enriched fraction separated on Ficoll-Hypaque.

Other Changes

After TPA treatment, the cells of all types of acute nonlymphocytic leukemia showed a decrease in the nuclear:cytoplasmic ratio, a coarsening of chromatin, the disappearance of specific granulations, the appearance of cytoplasmic vacuoles, a rounding or indenting of the nucleus, and the permanence of a nucleolus. The myeloid leukemic cells treated with TPA also showed a decrease in the number that stained positive in the Sudan Black and peroxidase reactions. Changes in PAS staining were observed in some cases, but without a well defined pattern. However, none of these changes were accompanied by morphologically recognizable differentiated granulocytes or monocytes. No morphological changes were found in TPA-treated lymphoblasts.

DISCUSSION

In this article, we report that in vitro treatment of human acute myeloid leukemia cells with TPA induces a number of morphological, functional, and histochemical changes within 72 hr characteristic of more differentiated cells. The effect of TPA is to drive cells in the direction of expressing the specific characteristics of monocytes and macrophages. Acute monocytic leukemia cells already display these characteristics, and little effect is seen with TPA in these cells. Lymphoblastic leukemia cells and some undifferentiated or unclassified leukemia cells do not undergo the series of changes seen with myeloid leukemia cells.

The effects of TPA on adhesion, phagocytosis, and specific staining observed in this study are similar to the effects observed with TPA in HL-60 cells. Preliminary results also indicate that TPA suppresses leukemic cell proliferation. All elements of the myeloid lineage, from the immature leukemic myeloblast to the more mature myelocyte, responded to TPA treatment in vitro. The effect of TAP on myeloid cells is not restricted to leukemic cells; we have some preliminary evidence that normal precursors also respond to TPA treatment. The cellular aggregation induced by TPA in several cases of lymphoblastic leukemia is similar to that reported to occur with TPA treatment of established lymphoblastoid cell lines. We did not examine whether markers of mature lymphocytes were induced by TPA, and further studies are needed.

Some undifferentiated or unclassified leukemias respond to treatment with TPA by developing adhesiveness and phagocytic ability; others undergo clumping. This suggests that the response of unclassified leukemias to TPA may assist in cell classification and selection of therapy.

The major conclusion from our studies is that the effect of TPA on leukemic cells is not unique to established myeloid leukemia cell lines but is a property of the majority of myeloid leukemias as they occur in patients. It suggests that the inability of leukemic cells to further differentiate in vivo may represent a failure to receive or to correctly process the physiologic signals for differentiation.
REFERENCES


NOTE ADDED IN PROOF

Two other cases of promyelocytic leukemia and one case of myeloblastic leukemia were found to be unresponsive to TPA treatment in vitro, thus confirming that sensitivity to TPA is not a constant finding in human myeloid leukemias.
Differentiation of human leukemias in response to 12-0-tetradecanoylphorbol-13-acetate in vitro

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