# Tumor Cell Killing by Phorbol Ester–Differentiated Human Leukemia Cells

Abstract. *The tumor promoter 12-O-tetradecanoyl-phorbol-13-acetate causes differentiation of cells of the human leukemia cell line HL60 to nondividing macrophage-like cells. These differentiated cells are cytotoxic for tumor cells (including parent, untreated HL60 cells) in vitro. Agents that induce this desirable differentiation to nondividing, antitumor effector cells may be useful in the experimental treatment of leukemia.*

Macrophages, after appropriate manipulation in vivo or in vitro, are cytostatic and cytocidal for tumorigenic target cells in vitro (*1*). Although most studies have been done in murine systems, human monocytes and macrophages are also capable of mediating tumor cell cytotoxicity in vitro (*2*). Such cytotoxic macrophages, which discriminate between malignant and nontransformed target cells (*3*), may act as the effector cells of a system capable of detecting and eliminating malignant cells in vivo (*4*). Recently, Rovera and coworkers (*5*) and Lotem and Sachs (*6*) demonstrated that cells of the stable human promyelocytic leukemia cell line HL60 (*7*), when cultured in vitro with the phorbol diester 12-*O*-tetradecanoylphorbol-13-acetate (TPA), differentiate into cells with macrophage characteristics. They stop dividing, become adherent and phagocytic, and develop increased cellular concentrations of the enzymes $NAD^+$ nucleosidase (E.C. 3.2.2.5), acid phosphatase, lysozyme, and nonspecific esterase (*5, 6*). I report here that these macrophage-like cells differentiated from TPA-treated HL60 cells are cytostatic and cytocidal for human malignant cells in vitro, whereas the undifferentiated HL60 cells have no effect on tumor cell growth.

The nonadherent HL60 cells were grown in suspension culture in 10 percent heat-inactivated fetal bovine serum or 10 percent heat-inactivated human serum with supplemented Dulbecco's modified Eagle's medium (DMM) (*8*). For treatments with TPA, the HL60 cells were incubated in suspension culture in 50-ml polypropylene tubes (Corning No. 25330). In these hydrophobic tubes, there was no adherence to the plastic despite the presence of TPA. After 3 days of culture with the desired additives, the cells were washed, placed into microtiter chambers 6 mm in diameter (Falcon No. 3040), and assayed for tumor cytotoxicity by visual cell counting and quantitation of release of [$^3$H]thymidine or $^{51}$Cr from labeled tumor cells (*1*).

As shown in Fig. 1, undifferentiated HL60 cells were noncytotoxic for the tumor cells. However, after the HL60 cells were cultured for 3 days with as little as 10 to 25 ng of TPA per milliliter, the macrophage-like cells were cytotoxic and cytocidal for cells from various established tumor cell lines including the human lines HeLa, HL60, HSB, trans-

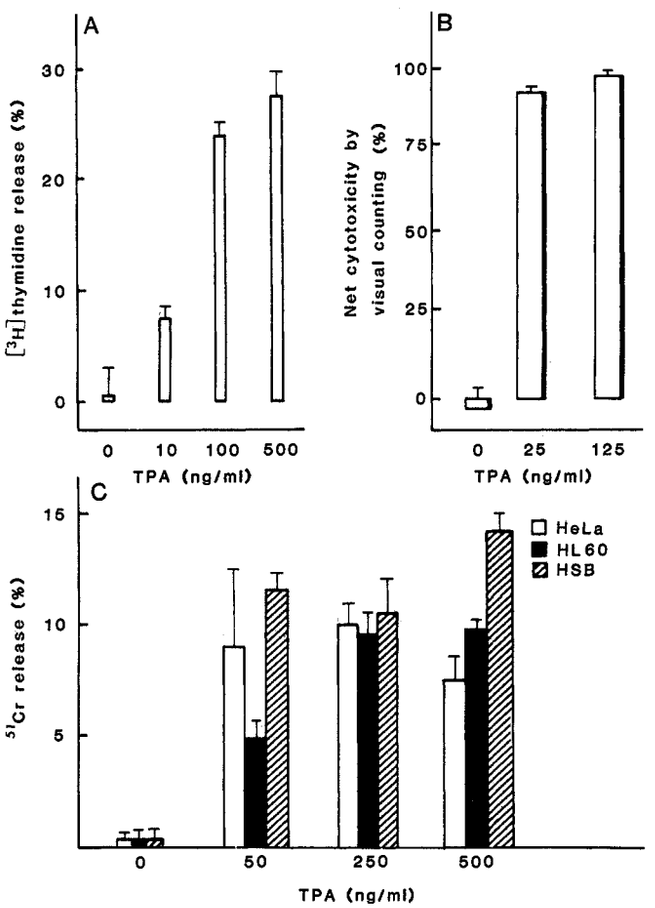


Fig. 1. Tumor cell cytotoxicity mediated by TPA-treated HL60 cells. HL60 cells were cultured in DMM with 10 percent fetal bovine serum and the designated amount of TPA for 3 days. They were washed two times with DMM, and $0.8 \times 10^5$ to $1.0 \times 10^5$ HL60 cells were placed in microtiter wells with $1.0 \times 10^4$ labeled tumor target cells in DMM containing 10 percent fetal bovine serum (total volume, 0.2 ml). (A) HeLa target cells were labeled with [$^3$H]thymidine as previously described (*11*). The percentage of release of [$^3$H]thymidine was determined after 60 hours of culture by counting cell-free supernatants and using the formula

$$([^3\text{H}]\text{thymidine})_{\%\ \text{released}} = \frac{(\text{sup.})_{\text{exp.}} - (\text{sup.})_{\text{contr.}}}{\text{total}} \times 100$$

where $(\text{sup.})_{\text{contr.}}$ is supernatant of HeLa cells alone (spontaneous release), $(\text{sup.})_{\text{exp.}}$ is supernatant of HL60 cells plus HeLa cells, and "total" is the total number of counts for HeLa cells determined by lysing with 0.2 percent sodium dodecyl sulfate. (B) Experiments with unlabeled HeLa cells. The percentage of net cytotoxicity (PNC) at 60 hours was determined by visually counting tumor cells and using the formula

$$\text{PNC} = 1 - \frac{(\text{HeLa/chamber})_{\text{exp.}}}{(\text{HeLa/chamber})_{\text{contr.}}} \times 100$$

where the $(\text{HeLa/chamber})_{\text{exp.}}$ refers to chambers of HeLa cells with HL60 cells, and $(\text{HeLa/chamber})_{\text{contr.}}$ refers to chambers of HeLa cells without HL60 cells (*10*). (C) Experiments with HeLa, HL60, and HSB cells labeled with $^{51}$Cr. Target cells ($2 \times 10^6$) were labeled at 37°C with 100 μCi of sodium dichromate (New England Nuclear, 200 to 500 Ci/g), washed two times with DMM, and added to wells with HL60 effector cells in DMM containing 10 percent fetal bovine serum (final volume, 0.2 ml). The percentage of release of $^{51}$Cr was determined after 18 hours of culture by counting cell-free supernatants and using formula

$$(^{51}\text{Cr})_{\%\ \text{release}} = \frac{(\text{sup.})_{\text{exp.}} - (\text{sup.})_{\text{contr.}}}{\text{total}} \times 100$$

where $(\text{sup.})_{\text{exp.}}$ is the supernatant of TPA-treated effector cells with target cells, $(\text{sup.})_{\text{contr.}}$ is the supernatant of control effector cells (TPA = 0) with target cells, and "total" is the total number of counts for target cells determined by lysing with 0.2 percent sodium dodecyl sulfate. Spontaneous release from [$^3$H]thymidine-labeled cells was less than 20 percent over 60 hours of culture and about 1 percent per hour from $^{51}$Cr-labeled cells.


0036-8075/81/0807-0655$1.00/0 

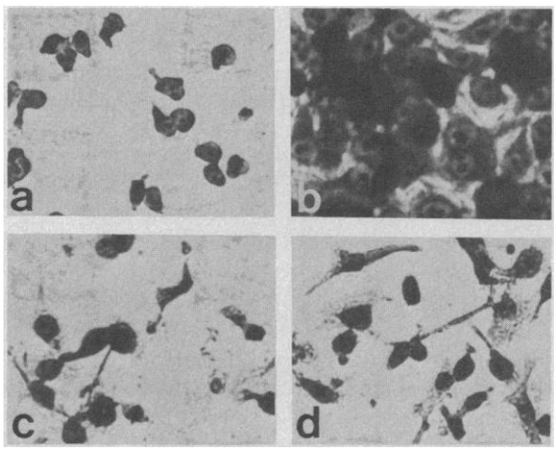


Fig. 2. Photomicrographs of HL60 cells with tumor cells. These photomicrographs were taken from microtiter chambers containing cells fixed and stained with Giemsa after 60 hours of culture. (a) HL60 cells not treated with TPA are round and not spread, whereas in (c), HL60 cells previously treated with TPA (100 ng/ml) are polarized and spread. (b) HeLa tumor cells overgrow the chambers containing untreated HL60 cells, but in (d), chambers containing TPA-treated HL60 cells show reduced numbers of HeLa cells. The microtiter chambers were seeded initially with $1 \times 10^5$ HL60 cells and $1 \times 10^4$ HeLa in 0.2 ml of DMM containing 10 percent fetal bovine serum. Magnifications, ×200.

formed WI38, and HEp2 and the mouse lines 3T12 and L cell (*9*). When the cells were counted visually (Fig. 1B), TPA-treated HL60 cells displayed great net cytotoxicity [a measure of cytostasis and cytolysis (*10, 11*)] for the tumor cells. In 18-hour assays in which the release of $^{51}Cr$ from labeled target cells was measured, TPA-treated HL60 cells lysed various tumor cells including the parent undifferentiated HL60 cells.

By microscopic examination of Giemsa-stained cells in microtiter chambers, the control, untreated HL60 cells were overgrown by the adherent tumor cells; in chambers with the TPA-treated HL60 cells, only rare tumor cells could be seen (Fig. 2). No phagocytosis of the tumor cells by the TPA-treated HL60 cells was seen. The cytotoxicity was present in cultures containing either fetal bovine serum or human serum (unheated or heated 30 minutes at 56°C). If the TPA-treated HL60 cells were depleted of the undifferentiated (nonadherent) cells before the cytotoxicity assay by using only those cells that adhered to microtiter chambers, the cytotoxicity of these macrophage-like cells was not diminished, indicating that the differentiated cells caused the effect. Although the TPA-treated HL60 cells were markedly cytotoxic for tumor cells, they expressed no or very little cytotoxicity for nonmalignant target cells. For example, HL60 cells previously treated with 100 ng of TPA per milliliter had a net cytotoxic effect of 96.8 ± 0.2 percent for HeLa cells, but only 24.2 ± 10.5 and 18.5 ± 1.2 percent for the normal fibroblasts Detroit 551 and WI38, respectively.

In agreement with studies of mouse macrophage–mediated tumor cytotoxicity (*1, 3*), the cidal effect appeared to be dependent on effector–target cell contact. If the TPA-treated HL60 cells had adhered unevenly to the bottoms of the microtiter chambers so that some areas were free of these effectors, the tumor target cells grew normally in the HL60 cell–free area whereas the tumor cell growth was dramatically inhibited in areas where the HL60 cells were present. If the medium was removed from chambers containing TPA-treated cells with HeLa cells and replaced with fresh medium every 6 to 12 hours during the 60-hour assay, the cytotoxic effect was not diminished. Supernatants from control or TPA-treated HL60 cells did not lyse labeled target cells; however, by visual counting, supernatants of TPA-treated cells caused mild stasis of HeLa cells (5 to 10 percent net cytotoxicity). Lysates produced by sonifying control or TPA-treated HL60 cells were not toxic for tumor cells. TPA alone did not cause lysis or stasis of target cells in the doses used here.

When cells from other human malignant hematopoietic cells lines [HSB, SB, and K562 (*12*)] were treated with TPA, they were cytotoxic for the HeLa cells. Whereas the T (HSB) and B (SB) lymphocyte cell lines manifested this TPA-induced cytotoxicity without evidence of change to macrophage-like cells, 3 to 8 percent of the K562 (chronic myelogenous erythroleukemia) cells became adherent and developed nonspecific esterase activity in response to the TPA treatment. These findings suggest that TPA may render some hematopoietic cell lines cytotoxic by mechanisms other than induction of differentiation to macrophage-like cells [possibly by actions related to its B- and T-cell mitogenic activity (*13*)]. TPA has been shown to enhance mouse macrophage–mediated tumor cell stasis or lysis in different systems (*14*). Likewise, TPA-treated human blood monocytes generally caused stasis and lysis of HeLa cells to a degree comparable to that caused by TPA-treated HL60 cells, whereas normal blood lymphocytes treated with TPA were generally not cytotoxic (*15*). Human red blood cells and red blood cell ghosts treated with TPA were not cytotoxic for HeLa cells.

These observations demonstrate that cells of a human leukemia cell line can differentiate in vitro to cells capable of killing their parent, undifferentiated leukemia cells as well as other established human tumor cell lines. The ability to grow large numbers of this relatively uniform population of cells provides a useful system for studies of membrane changes associated with this TPA-induced functional change, studies of controls of the differentiation to these tumoricidal macrophage-like cells, and studies of the actual molecular mechanisms of the nonphagocytic, contact-dependent tumor cell killing process.

Various agents cause differentiation of myeloid leukemia cells in vitro (*7, 16*); also, other workers have demonstrated that another human myeloid leukemia cell line (KG-1) and freshly isolated human acute nonlymphoid leukemia cells differentiate to macrophage-like cells when cultured in vitro with TPA (*17*). I speculate that, in addition to its enhancing effect on the HL60 tumoricidal activity demonstrated here, TPA may also have this desirable effect on other acute nonlymphoid leukemia cells in vitro. The leukemia cells that differentiate to macrophage-like cells could then act as effectors to destroy the few leukemia cells that escape the differentiating effects of TPA (*5, 6, 17*). HL60 cells, like many other cells, have specific high-affinity cell membrane receptors for biologically active phorbol esters (*18*). If endogenous ligands for these receptors were present [comparable to endorphin ligands for opiate receptors (*19*)], they might favorably influence leukemic cell differentiation and growth. These natural ligands would offer a potential avenue for the experimental treatment of leukemia—treatment in vivo with substances capable of inducing differentiation of the malignant leukemia cells to nondividing effector cells with antileukemic activity.

J. BRICE WEINBERG
*Division of Hematology-Oncology, Department of Medicine, VA Medical Center,* and *Duke University Medical Center, Durham, North Carolina 27705*

### References and Notes

1. P. Alexander and R. Evans, *Nature (London) (New Biol.)* **232**, 76 (1971); J. B. Hibbs, Jr., L. H. Lambert, Jr., J. S. Remington, *ibid.* **235**, 48 (1972); J. B. Hibbs, Jr., R. R. Taintor, H. A. Chapman, Jr., J. B. Weinberg, *Science* **197**, 279 (1977); S. W. Russell, W. F. Doe, A. T. McIntosh, *J. Exp. Med.* **146**, 1151 (1977).
2. J. J. Rinehart, P. Lange, B. J. Gormus, M. E. Kaplan, *Blood* **52**, 211 (1978); J. J. Rinehart, R. Vessella, P. Lange, M. E. Kaplan, *J. Lab. Clin. Med.* **93**, 361 (1979); D. J. Cameron and W. H. Churchill, *J. Clin. Invest.* **63**, 977 (1979); G. Unsgaard, J. Hammerstrom, J. Lamvik, *Acta Pathol. Microbiol. Scand. Sect. C* **87**, 159 (1979); A. Mantovani, T. R. Jerrells, J. H. Dean, R. B. Herberman, *Int. J. Cancer* **23**, 18 (1979); A. F. LoBuglio, M. J. Solvay, S. J. Weiss, *Blood* **54** (Suppl. 1), 103a (1979).
3. J. B. Hibbs, Jr., L. H. Lambert, J. S. Remington, *Nature (London) New Biol.* **235**, 48 (1972); M. S. Meltzer, R. W. Tucker, K. K. Sanford, E. J. Leonard, *J. Natl. Cancer Inst.* **54**, 1177 (1975).
4. J. B. Hibbs, Jr., H. A. Chapman, Jr., J. B. Weinberg, *J. Reticuloendothel. Soc.* **24**, 549 (1978).
5. G. Rovera, T. G. O'Brien, L. Diamond, *Science* **204**, 868 (1979); G. Rovera, D. Santoli, C. Damsky, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 2779 (1979); A. Vorbrodt, P. Meo, G. Rovera, *J. Cell Biol.* **83**, 300 (1979).
6. J. Lotem and L. Sachs, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 5158 (1979).
7. S. J. Collins, R. C. Gallo, R. E. Gallagher, *Nature (London)* **270**, 347 (1977); R. Gallagher, S. Collins, J. Trujillo, K. McCredie, M. Ahearn, S. Tsai, R. Metzgar, G. Aulakh, F. Ruscetti, R. Gallo, *Blood* **54**, 713 (1979).
8. The HL60 cells were obtained from R. Metzgar, Duke University Medical Center. They were grown by seeding at $5 \times 10^5$ cells per milliliter of DMM supplemented with dextrose (1 mg/ml), streptomycin (100 μg/ml), penicillin (100 U/ml), and 20 m*M* Hepes buffer. The medium was endotoxin-free as assessed by the *Limulus* amebocyte lysate assay (*11*). Maintenance cultures were subcultured two times per week with endotoxin-free fetal bovine serum (Sterile Systems, Inc., Logan, Utah).
9. The cells HeLa, HEp2, transformed WI38, normal WI38, and Detroit 551 were from American Type Culture Collection; the mouse cell lines are maintained in continuous serial passage in this laboratory (*1*, *4*); HSB, SB, and K562 were from R. Metzgar.
10. J. B. Weinberg and J. B. Hibbs, Jr., *J. Natl. Cancer Inst.* **63**, 1273 (1979).
11. J. B. Weinberg, H. A. Chapman, Jr., J. B. Hibbs, Jr., *J. Immunol.* **121**, 72 (1978); L. P. Ruco and M. S. Meltzer, *ibid*, p. 203.
12. R. A. Adams, L. Pothier, A. Flowers, H. Lazarus, S. Farber, G. E. Foley. *Exp. Cell Res.* **62**, 5 (1970); J. K. Anderson and R. S. Metzgar, *J. Immunol.* **120**, 262 (1978); C. B. Lozzio and B. B. Lozzio, *Blood* **45**, 321 (1975); L. C. Andersson, K. Nilsson, C. G. Gahmberg, *Int. J. Cancer* **23**, 143 (1979).
13. J. Touraine, J. W. Hadden, F. Touraine, E. M. Hadden, R. Estensen, R. A. Good, *J. Exp. Med.* **145**, 460 (1977); J. Abb, G. J. Bayliss, F. Deinhardt, *J. Immunol.* **122**, 1639 (1979).
14. C. F. Nathan, L. H. Bruckner, S. C. Silverstein, Z. A. Cohn, *J. Exp. Med.* **149**, 84 (1979); W. Grimm, E. Bärlin, H. Leser, W. Kramer, D. Gemsa, *Clin. Immunol. Immunopathol.* **17**, 617 (1980).
15. Normal human blood monocytes and lymphocytes were prepared to ≥ 95 percent purity as previously described [K. Kumagai, K. Itoh, S. Hinums, M. Tada, *J. Immunol. Methods* **29**, 17 (1979)]. The monocytes and lymphocytes were then cultured with or without TPA and assayed for cytotoxicity as described in the legend to Fig. 1.
16. L. Sachs, *Nature (London)* **274**, 535 (1978); S. J. Collins, A. Bodner, R. Ting, R. C. Gallo, *Int. J. Cancer* **25**, 213 (1980).
17. H. P. Koeffler, M. Bar-Eli, M. Territo, *Blood* (Suppl. 1) **54**, 174a (1979); *J. Clin. Invest.* **66**, 1101 (1980); L. Pegoraro, J. Abrahm, R. A. Cooper, A. Levis, B. Lange, P. Meo, G. Rovero, *Blood* **55**, 859 (1980).
18. P. E. Driedger and P. M. Blumberg, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 567 (1980); M. Shoyab and G. J. Todaro, *Nature (London)* **288**, 451 (1980).
19. J. Hughes, T. W. Smith, H. W. Kosterlitz, L. A. Fothergill, B. A. Morgan, H. R. Morris, *ibid.* **258**, 577 (1975).


20. This investigation was supported by the Veterans Administration and by NIH grant 27070-02. I thank M. Naramore, L.-V. Johnson, P. Terrell, J. Henderson, and D. Powell for technical assistance and J. Niedel for helpful discussions.

2 July 1980; revised 25 March 1981


## Structural Changes at the Heme Induced by Freezing Hemoglobin


Abstract. *A dramatic change occurs in the vibrational properties of the iron-histidine bond,* trans *to the oxygen binding site, on freezing deoxyhemoglobin. The large, quaternary structure–dependent differences in the shape and frequency of the iron-histidine mode observed in resonance Raman scattering measurements above freezing are significantly diminished by the freezing event and the scattering intensity increases substantially. On further reduction in temperature to 10 K this broad line becomes narrow and shifts to a higher frequency. These data implicate dynamical processes and protein interaction with water as contributors to the quaternary structure dependence of the iron-histidine bond and thus reflect on the role of that bond in the energetics of cooperative ligand binding.*


Resonance Raman scattering studies of deoxyhemoglobins and deoxymyoglobins have led to assignment of the mode at 216 $cm^{-1}$ to stretching of the bond between the heme iron and the proximal histidine nitrogen (*1–4*). The properties of this mode under various states of ligation and different protein conformations will ultimately reveal the role played by the proximal histidine in controlling the energetics of cooperative ligand binding. Large differences in the frequency and shape of this mode were found when T-state human adult hemoglobin (HbA) was compared with R-state NES des-Arg-hemoglobin under solution condition at or slightly below room temperature (*4–6*). Nagai and Kitagawa (*5*) also reported recently that in T-state valency hybrid hemoglobins the α chains display a different frequency for this mode than do the β chains. In measurements at a very low temperature one might expect that the lines would narrow and one could thereby distinguish the contributions from the α and β chains in native HbA. We therefore made resonance Raman scattering measurements of this iron-histidine mode in HbA over the temperature range 10 to 300 K. We found that in the measurements on frozen samples at a very low temperature (10 K) the quaternary structure–dependent differences in this mode that are seen at room temperature are completely absent. On warming the samples from 10 K to just below freezing, the iron-histidine stretching mode in hemoglobins stabilized in both quaternary structures broadens and shifts to a lower frequency. However, the R-T differences in this mode develop only on going through the melting transition at 0°C.

The Raman measurements on solution samples from 25° to 0°C or on frozen samples from 0° to −20°C were made with Raman difference instrumentation with which very small differences (< 0.1 $cm^{-1}$) may be detected by simultaneously comparing two samples (*7*). The liquid samples were placed in a split rotating cell and frozen by a stream of cold nitrogen gas. Measurements on individual samples below 200 K were made with the same spectrometer without using the rotating cell. For these measurements small drops of sample were placed on a precooled cold finger of an Air Products Heli-Tran refrigerator, followed by evacuation and then adjustment to a selected temperature. Spectra were obtained with excitation frequencies of 5287 and 4579 Å from an argon ion laser and 4131 Å from a krypton ion laser. Samples were stored either at 4°C or in liquid nitrogen and were chromatographed on a column before use. The procedure of Kilmartin and Hewitt (*8*) was used to prepare NES des-Arg-HbA. A solution of 1 m*M* HbA in 10 to 80 m*M* sodium phosphate buffer (*p*H 7.0) was found to yield nonfluorescent samples for low-temperature studies.

The Raman mode at 216 $cm^{-1}$ in HbA has been assigned as the iron-histidine stretching frequency on the basis of isotope and model compound studies (*2–4*, *6*). It displays an asymmetric line shape in T-state deoxy HbA and becomes more intense, symmetric, and shifts to higher frequency in R-state deoxy-NES des-Arg-HbA. This behavior was previously reported (*4–6*) and is illustrated in Fig. 1 (see spectra a and c). Other small differences in the low-frequency region between native HbA and the R-structure chemically modified form have also been reported (*6*). Differences in bands sensitive to electron density between 1200 and 1650 $cm^{-1}$ have been detected as well, and interpreted as evidence for charge transfer interactions between the porphyrin macrocycle and amino acid residues of the globin (*9*).



0036-8075/81/0807-0657$01.00/0