Differential Effects of Phorbol Esters on Normal Myeloid Precursors and Leukemic Cells: Basis for Autologous Bone Marrow Reconstitution in Acute Nonlymphocytic Leukemia Using Phorbol Ester-treated Bone Marrow from Patients in Remission

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ABSTRACT

The phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) induces macrophage-like differentiation of HL60 cells and cells from patients with acute nonlymphocytic leukemia (ANLL). We assessed the use of TPA as a means of eradicating residual leukemia from remission bone marrow prior to autologous bone marrow reconstitution. A 30-min incubation with TPA led to marked growth arrest in HL60 cells and in cells from most patients with acute myelogenous leukemia and acute myelomonocytic leukemia, whereas cells from most patients with acute promyelocytic leukemia and acute undifferentiated leukemia demonstrated a lesser degree of growth arrest. Freezing and thawing, a necessary step in autologous reconstitution, had no effect on the cessation of proliferation induced in HL60 or ANLL cells preincubated with TPA for 30 min. Virtually normal myeloid precursor growth occurred in normal or remission bone marrow cells preincubated with TPA and then frozen and thawed. Based on these observations, two patients with advanced ANLL in remission underwent marrow ablative therapy followed by autologous reconstitution using TPA-treated bone marrow. Limited normal hematopoiesis was reestablished in both patients, although they subsequently experienced leukemic relapse. These studies demonstrate that in ANLL cells, TPA stimulates growth arrest; in contrast, hematopoiesis is able to proceed both in vitro and in vivo.

INTRODUCTION

Phorbol esters cause HL60 (1, 2) and myeloid leukemia cells (3, 4) to cease proliferation and to undergo differentiation into cells resembling mature macrophages. The best defined mechanism of phorbol ester action is through its specific binding to cells (5, 6) and activation of protein kinase C (7). PDB is a relatively hydrophilic phorbol ester that can be removed from its cellular binding sites by washing (6). Removal of PDB halts the differentiation program (6), indicating that the continued association of phorbol esters with cells is critical during the maturation induction process. In contrast to PDB, TPA is hydrophobic. When cells are washed free of external TPA, differentiation continues and proliferation ceases (8).

Phorbol esters also affect normal myelopoiesis in vitro. This effect is complex. For example, in the absence of an exogenous source of CSA, phorbol esters directly stimulate GM-CFU, with a disproportionate stimulus of macrophage colony growth (9, 10); however, when adherent non-colony forming cells obtained from normal bone marrow are incubated with phorbol esters, they elaborate a form of CSA which preferentially stimulates the growth of granulocyte colonies (11). In contrast, in the presence of exogenous CSA from a variety of sources, phorbol esters inhibit granulocyte colony growth (9, 12). Other cells resident in human bone marrow are also influenced by phorbol esters. For example, phorbol esters stimulate the proliferation of T-lymphocytes, resulting in interleukin 2 and CSA production in the absence of interleukin 1 (13); moreover, when combined with other stimulatory factors, phorbol esters stimulate B-lymphocyte proliferation (14).

The fact that phorbol esters can induce cell differentiation and halt cell proliferation in myeloid leukemia cells while not preventing the growth of normal GM-CFU suggested that certain phorbol esters might have a therapeutic role; however, the multiplicity of effects which phorbol esters have on various normal tissues including the activation of circulating granulocytes (15, 16) and platelets (16, 17) precluded consideration of their therapeutic use systemically; therefore, we reasoned that TPA could be added to bone marrow harvested from patients with ANLL in remission with a goal of selectively affecting the residual leukemic cell population while preserving the normal proliferative pool of hematopoietic precursors. Others have tried a similar strategy to eradicate the residual malignant cells, using treatment of marrow with drugs highly selective for the malignant process (18), use of monoclonal antibodies that bind malignant cells (19, 20), or use of immunotoxins consisting of monoclonal antibodies conjugated to ricin (21). Used in this fashion, TPA might permit autologous reconstitution after intensive chemo- and radiation therapy during the remission period of ANLL.

The present report includes 3 phases of our work toward this end: (a) we have examined the ability of TPA to continue to affect HL60 cells, ANLL cells, and GM-CFU after its removal from the incubation medium; (b) we have examined the biological effects of TPA in leukemic cells under the conditions of incubation and cryopreservation used in autologous marrow reconstitution; (c) data are presented which demonstrate that TPA-treated remission bone marrow was capable of reconstituting hematopoiesis in two patients with advanced ANLL following marrow ablation with TBI and chemotherapy.

MATERIALS AND METHODS

Chemicals. PHA (PHA HA-15; Burroughs-Wellcome, Greenville, NC) was reconstituted in distilled water and stored at −80°C until used.
TPA (Life Systems, Newton, MA) was reconstituted at 10^2 or 10^3 M in acetone, stored at —80°C, and diluted in culture medium immediately before use. Sterile powdered TPA (Consolidated Midland Corp., Brewster, NY) for use only in autologous marrow treatment was dissolved in acetone at a concentration of 10^3 M solution, maintained at —80°C, and diluted immediately before use. [3H]dThd (1 mCi/ml) was obtained from New England Nuclear (Boston, MA).

Cell Culture. HL60 cells (provided by Dr. Robert Gallo, National Cancer Institute) were grown in plastic tissue culture flasks (Corning Glass Works, Corning, NY) in RPMI 1640 containing 15% (v/v) fetal calf serum (Flow Laboratories, Inc., McLean, VA) and supplemented with 20 mM L-glutamine-1% vitamins-1% penicillin/streptomycin (10,000 units/ml) (Flow). For morphological studies, including histochemical staining for nonspecific esterase (22), cells were grown in plastic wells (Costar, Cambridge, MA) with glass coverslips.

Freezing. HL60 or freshly obtained leukemic or normal marrow cells were diluted to 20 x 10^6 cells/ml in RPMI 1640 supplemented with 20% autologous or bovine serum, mixed with equal vol of 20% DMSO (Fisher), frozen in a programmable freezer to —80°C, and diluted in culture medium immediately before use. Sterile powdered TPA (Consolidated Midland Corp., Brewster, NY) was reconstituted at 10^2 or 10^3 M and diluted immediately before use. [3H]dThd (1 mCi/ml) was obtained from New England Nuclear (Boston, MA).

In order to measure DNA synthesis in HL60 cells and with bone marrow or peripheral blood leukemic cells from 63 patients. They included the following subgroups of acute leukemia: AML (18), ANML (17), AMOL (3), APL (6), AUL (10) and ALL (9). HL60 cells were reconstituted at 2 x 10^6/ml in RPMI 1640 alone or in medium containing 10^-6 M TPA, and unless otherwise indicated, they were incubated for 30 min at room temperature in room air. Following incubation, cells were washed 3 times and then either frozen or resuspended at 2 x 10^6 cells/ml for studies of DNA synthesis. In some early experiments, patients' leukemic cells were incubated in the continuous presence of 5 x 10^-8 M TPA rather than being exposed briefly to 10^-6 M TPA. DNA synthesis was similar under these two conditions, and the results are presented together.

For measurement of DNA synthesis, 200 /l of the cell suspension were placed in each of 4-5 wells of 96-well microtiter plates (Costar) and incubated 0-7 days at 37°C and 5% CO2. On the day of harvest, 50 /l of RPMI 1640 containing [3H]dThd (20 /lCi/ml) were added, and the cells were incubated for 2 h at 37°C and 5% CO2 and transferred to Whatman glass fiber filters using a cell harvester (Brandel, Gaithersburg MD). The dried filters were cut into two halves containing ScintiVerse for determination of radioactivity in a liquid scintillation counter. Responders were defined as those patients whose TPA-treated cells incorporated >10% of [3H]dThd taken up by their control untreated cells.

Monoclonal Antibody Analysis of Surface Antigen Profile. Monoclonal antibody diluted in staining buffer (phosphate buffered saline with 0.1% sodium azide and 2% bovine serum albumin) was added to 0.25-1.0 x 10^6 cells suspended in 100 /l of RPMI 1640. After vigorous mixing, the cells were incubated on ice for 30 min, resuspended in 25 /l of fetal calf serum and 25 /l of fluorescein isothiocyanate-conjugated goat anti-mouse F(ab')2 (Tago, Inc., Burlingame, CA), mixed, incubated for 30 min on ice, and washed to remove unbound fluorescent antibody. The cells were then washed and resuspended in a 10^-6 M solution of the antibody of interest. For the purposes of this study, no further antibody was added.

The antibody panel used included: P3, mouse myeloma protein from ascitic fluid (prepared by Dr. R. Kenne); OKT3, OKT4, OKT8, OKT9, OKT10, OKT11 (Ortho Diagnostics, Raritan, NJ); OKLa (Ortho); OKM1 (Ortho); 63D3 (Bethesda Research Laboratories, Bethesda, MD), and either P155.3.3 (kindly provided by Dr. R. Kenne) or J5 (Coulter Immunology, Hialeah, FL).

Normal Myelopoiesis after TPA Exposure. Ficoll-Paque interface bone marrow cells from eight normal healthy donors and from two autologous marrow transplant candidates in remission were washed and suspended in RPMI 1640 supplemented with 15% fetal bovine serum (Flow). One-half of each sample was incubated in medium alone with 0.1% acetone and the other half in medium containing 0.1% acetone and 10^-6 M TPA for 30 min at room temperature in room air. Both control and TPA-treated cells were then washed with plain medium three times and either frozen or placed directly in semisolid agar cultures.

Cultured cells were incubated, fixed, and stained for histochemical markers of granulocytes and monocytes as previously described (9). Briefly, fresh or thawed cells were added to a mixture of Dubecco's modification of Eagle's medium (Flow) modified according to the method of Metcalf (23) with 20% fetal calf serum and 0.3% Bacto-Agar (Difco Laboratories, Detroit, MI) to a final concentration of 10^6 cells/ml; thereafter, 1 ml of the cell suspension was placed in 35 cm² gridded Petri dishes (Lux Lab-Tek Division, Miles Laboratories, Naperville, IL) to which 100 /l stimulating medium had been added. Medium conditioned by normal human peripheral blood leukocytes exposed to 1% (v/v) PHA according to the method of Aye (24) was used as the source of colony stimulating activity. After gelling, the cultures were incubated up to 14 days in a 10% CO2 fully humidified atmosphere at 37°C. They were fixed with formal calcium, transferred to 3- x 2-inch glass slides, dried under cellulose acetate filter papers (Schleicher and Schuell, Inc., Keane, NH), and then stained for chloroacetate and a-naphthylacetate esterases by the method of Yam et al. (22), as modified by Abraham and Smiley (9). Duplicate or quadruplicate cultures were performed. Groups of greater than 20 cells were considered colonies. Colonies were considered granulocytic if more than 90% of the cells in the colony contained chloroacetate esterase, monocyte if more than 90% of the colony cells contained a-naphthylacetate esterase, and mixed if neither enzyme predominated by 90%. For data analysis, Student's t test for paired samples was used.

Autologous Transplantation Protocol. Patients' eligibility requirements for autologous bone marrow reconstitution with TPA-exposed marrow included confirmed diagnosis of ANLL in second or third remission, age 18-60 years, previous documentation that proliferation of leukemic cells was decreased >95% after TPA exposure as measured by [3H]dThd incorporation, and no medical contraindications. The protocol was approved by the university's Committee on Studies Involving Human Subjects and by the Food and Drug Administration.

The two patients studied gave informed consent. After demonstration of normal bone marrow GM-CFU growth in the thawed TPA-treated marrow aspirate from each patient, the patient's marrow was harvested (25). Nine hundred and ninety ml of marrow from patient 1 and 1270 ml from patient 2 were removed from multiple bilateral iliac crest aspirates. The marrow cell suspension was centrifuged at 2500 x g for 10 min, the buffy coat was removed, and 3-4 x 10^6 cells were frozen to serve as rescue in the event of inadequate repopulation by TPA-exposed marrow.

The remaining cells were layered over Ficoll-Paque and centrifuged to 800 × g for 30 min at 22°C. The interface cells were washed and resuspended in heparinized RPMI 1640 media containing 10^-6 M TPA. These cells and 4 x 10^6 control cells (not exposed to TPA) were incubated in room air at room temperature for 30 min, washed three times, and frozen. Aliquots of TPA-treated and untreated marrow were placed immediately into agar culture. The next day a pilot bag containing TPA-treated marrow was thawed and cultured. GM-CFU content of the frozen TPA-treated marrow was calculated from the in vitro marrow colony growth at 10 days of incubation corrected for cell recovery. In both patients, GM-CFU content was adequate to suggest successful reconstitution (26). Seventeen (patient 1) and 18 days (patient 2) after marrow harvesting, the patients underwent standard marrow ablative treatment using cyclophosphamide (60 mg/kg/day on days —6 and —5) and TBI in three 333-rad fractions at 8 rads/min on days —3, —2, and —1. Twenty-four h after the patient completed TBI, the TPA-exposed remission marrow was rapidly thawed at 37°C and immediately reinfused.

Patient Characteristics. Both patients were selected for study because they had demonstrated brief remissions and were in second and third...
remissions, respectively, thus offering little opportunity for response to conventional therapy. Although in remission at the time of study, it was recognized that both probably had a significant leukemic cell burden. Patient 1 was a 48-year-old white male with AML who presented initially with chloromas and a WBC of 90,000/mm³. Chromosome studies were unsuccessful. Remission was achieved after two courses of DAT complicated by leukemic meningitis and by pneumonia of uncertain etiology. Consolidation therapy with AraC/AMSA was complicated by Aspergillus pneumonia treated with amphotericin B. Relapse, 8 months later, was treated with Mitoxantrone (12 mg/m²/day i.v. for 5 days) producing a second remission. Two months later marrow was harvested. Marrow biopsy at that time contained one focus of leukemic cells against a background of normal-appearing bone marrow. After cyclophosphamide and TBI, TPA-exposed marrow containing a calculated 112 x 10⁴ GM-CFU was reinfused.

Patient 2 was a 39-year-old white female with AMML. Chromosome evaluation revealed a 46,XX(11q−) pattern. Remission was achieved after two courses of DAT, and the patient underwent AraC/AMSA consolidation complicated by septic shock, acute renal failure, and both candidal and herpes esophagitis. The first relapse occurred 5 months later, and a second remission was achieved with Mitoxantrone as described above. A second relapse occurred within 6 weeks, and a third remission was achieved 3 months later with cyclophosphamide, vincristine, 1-ß-D-arabinofuranosylcytosine, and prednisone therapy (27). One month later, marrow harvest was performed. A single nonsecreting granuloma was seen on bone marrow biopsy, and no leukemic cells were identified. After cyclophosphamide and TBI, TPA-exposed marrow containing a calculated 74 x 10⁴ GM-CFU was reinfused.

RESULTS

DNA Synthesis in TPA-treated Leukemic Cells. Rovera et al. (1) demonstrated that HL60 cells incubated with 1.6 x 10⁻⁷ M TPA not only developed characteristics of mature macrophages but also ceased proliferating, as assessed by [³H]dThd incorporation. Studies in our laboratory had suggested that brief exposure to a higher concentration of TPA might be equally effective. Specific binding studies after a 30-min exposure of HL60 cells to 10⁻⁶ M TPA demonstrated occupancy of more than 90% of the phorbol binding sites as well as a large pool of cell-associated TPA; furthermore, occupancy of these sites led to cell adherence and production of α-naphthylacetate esterase, a monocyte marker (22), in greater than 90% of cells. In contrast, a 30-min exposure to 10⁻⁶ M TPA led to occupancy of only one third as many sites and no development of adherence or esterase activity. HL60 cells (10⁶-10⁸ cells/ml) were therefore incubated for 30 min with 10⁻⁶ M TPA, washed, and subsequently incubated up to 7 days to assay the effect of TPA on cellular DNA synthesis. Incorporation of [³H]dThd reached immeasurable levels between 24 and 48 h and remained there for the subsequent 5 days of the experiment. The effect of brief exposure of human leukemia cells to 10⁻⁶ M TPA depended on the cell type. TPA treated AML or AMML cells (M1 + M2 or M4, respectively, classified by French-American-British Cooperative Group criteria) (28) displayed a marked decrease in DNA synthesis. The median among AML patients was 6.5% of control values, and among AMML patients it was 5.5% of control values (Fig. 1). AMML cells responded (i.e., decreased [³H]dThd incorporation to <10% of that of control cells) most consistently. In ANLL cells responsive to treatment with TPA, DNA synthesis ceased by 24 h of incubation and did not resume over the subsequent 7 days of study (Fig. 2), whereas DNA synthesis of untreated control cells continued to increase. ALL cells exposed to TPA, however, increased their DNA synthesis at 24 h to a median value which was 2-fold that of untreated leukemic controls (Fig. 1).

To test whether increasing the duration of TPA exposure would affect ANLL cells which were unaffected by exposure to TPA for 30 min, the duration of the incubation with TPA was increased (Fig. 3). ANLL cells which were refractory to a 30-min TPA exposure did not cease proliferation even after exposure to 10⁻⁶ M TPA for up to 16 h. Responders' DNA synthesis remained at <10% of control, whereas nonresponders' synthesis remained at control levels in 2 cases and increased in a third case.

The presence or absence of an abnormal karyotype in ANLL cells did not predict responsiveness to TPA treatment. Chromosome analysis was performed by Dr. P. Nowell in 17 of 19 AML, 16 of 17 AMML, 8 of 9 ALL, and all patients with APL, AMoL, and AUL. TPA decreased DNA synthesis in the cells of 12 of 27 (44%) patients with ANLL with abnormal chromosomes and in 10 of 18 (55%) with entirely normal karyotypes. Of cells with abnormal karyotypes, the two AMLs

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Footnote: ⁶ Unpublished observations.
with t(8;21), the two APLs with t(15;17), and 3 of the 4 leukemic cells with −7 or −7q were unresponsive to TPA treatment.

Monoclonal antibody analysis of surface antigens was performed in 14 of 19 AML, 13 of 17 AMML, 2 of 3 AMoL, 5 of 6 APL, 7 of 10 AUL, and 8 of 9 ALL patient cells. In general, TPA effects on DNA synthesis correlated with classification by French-American-British Cooperative Group criteria, and the surface antigen profile of AML and AUL cells did not add to the ability to predict which cells would respond to TPA. Absence of HLA-DR antigen, however, was associated with nonresponse in 4 of the 5 APL patients studied. The only APL responder lacked the t(15;17) translocation and demonstrated the OKM1 surface antigen; in addition, while 5 of 9 AMML and 1 of 1 AMoL responders had the (HLA-DR+, OKM1+, 63D3+) profile, this profile was seen in only 1 of 4 AMML nonresponders, and it was not seen in the one AMoL nonresponder.

To assess the effect of TPA exposure on DNA synthesis in normal myeloid precursors, Ficoll-Hypaque interface marrow cells from two normal donors were similarly incubated with TPA or plain media and uptake of [3H]dThd was measured immediately and at 2, 4, and 7 days later. By day 4, [3H]dThd uptake of TPA-treated cells from both donors had returned to the level seen in untreated cells (data not shown).

Effect of Freezing and Storing on Inhibition of DNA Synthesis in HL60 and ANLL Cells Pretreated with TPA. Since TPA-treated marrow must be frozen, thawed, and reinfused, we questioned whether the phase changes associated with freezing and thawing cells in the presence of DMSO would affect cell-associated TPA. Freezing and thawing had no effect on the growth inhibitory effects of TPA in HL60 cells exposed to TPA for 30 min prior to freezing (data not shown); similarly, as seen in fresh ANLL cells (Fig. 2), there was no DNA synthesis in TPA-pretreated frozen and thawed cells from 2 ANLL patients, whereas DNA synthesis continued to increase in thawed control cells during 4 days of incubation (at day 4, patient 1 control cells, 160,000 ± 20,000 (SD) cpm/10^6 cells; patient 2 control cells, 90,000 ± 25,000 cpm/10^6 cells); moreover, there was no resumption of DNA synthesis in thawed TPA-pretreated ANLL cells after 8 days in culture whereas DNA synthesis continued to increase in the control cells of one patient and decreased by 50% in the cells of another patient.

Normal and Remission GM-CFU Growth after TPA Exposure. In addition to decreasing leukemic cell growth, TPA treatment must permit normal marrow growth if autologous reconstitution with TPA-treated marrow is to be successful. Our earlier studies (9) of the effect of TPA on GM-CFU did not reproduce the conditions of TPA treatment of marrow to be reinfused. In these earlier experiments, nonadherent Ficoll-Paque interface cells were grown with various concentrations of TPA continuously present in the agar cultures. In the current experiments, marrow cells were treated similarly to those to be reinfused after incubation with TPA. Adherent cells were not removed, and TPA exposure was continued for only 30 min, after which TPA was removed from the media by washing.

Possibly because of enrichment of precursors, more myeloid colonies grew from thawed normal or remission marrow than from marrow freshly placed into culture (Fig. 4). The difference was most striking in the remission cultures. Although cell counts of thawed normal and remission cells were similar, it is possible that remission marrow contained inhibitors of GM-CFU growth which were inactivated by the freeze-thaw procedure.

In addition, TPA-treated fresh normal marrow grew much less well than untreated marrow. This may have resulted from release of granular and nuclear material from TPA-treated granulated cells (myelocytes, metamyelocytes), which caused either an increased cytotoxic effect on GM-CFU or decreased removal of non-cell-associated TPA than occurred with TPA-treated HL60 cells. There was much less difference in colony growth between thawed control and TPA-treated aliquots. This successful growth may have been due to partitioning of the lipid soluble TPA into the DMSO (10% of final vol) included in the freezing media and the subsequent removal of both DMSO and TPA by washing. There was, however, a significant decrease (P < 0.05) in granulocyte colonies grown from the TPA-treated aliquot, indicating that a persistent TPA effect was observed after the freeze-thaw procedure.

To determine whether normal marrow would tolerate exposure to higher concentrations of TPA, cells from 3 normal subjects were treated with 10^-3 M TPA or incubated in media which included the TPA diluent, 0.1% acetone for 30 min, then

![Fig. 3. Effect of increased duration of exposure to TPA on DNA synthesis in responsive and unresponsive ANLL cells. ANLL cells known to be responsive or unresponsive to 10^-4 M TPA for 30 min were processed as described in legend to Fig. 1, except that the duration of exposure to TPA (abscissa) was increased to 1, 2, or 16 h before the cells were washed. [3H]dThd (TdR) uptake (ordinate) was determined at 16 h. Data were calculated from the mean of each individual's quadruplicate cultures. Mean ± SD (bars) of 4 responders and 3 nonresponders is shown.](image-url)

![Fig. 4. Effect of freezing on the growth of GM-CFU from normal and remission bone marrow that has been pretreated with TPA. Untreated (C) and TPA-treated (10^-4 M TPA for 30 min at room temperature) marrow from 8 normal donors (left) was either placed fresh into culture or frozen, thawed, and cultured as described in "Materials and Methods." Frozen and thawed marrow samples from the two patients who received autologous marrow infusions were also studied (right). The mean ± SE (bars) of granulocyte (C), monocyte (B), or mixed (D) colonies per 10^6 cells plated are shown. Mean data for the group were calculated using the mean from each individual's triplicate cultures.](image-url)
RECONSTITUTION IN ANLL USING TPA-TREATED CELLS

Changes in cell morphology and markedly decreases DNA synthesis in fresh or thawed cells. The responsiveness of blast cells morphologically identifiable as AMML together with the unresponsiveness of most APL and AML cells suggests that some maturation towards the monocytic pathway may be required for cells to be sensitive to the inducing effect of TPA. Both cell surface antigen profile and chromosome analysis provide support for this hypothesis. The 63D3 antigen, found on circulating monocytes, was not found on AML cells or unresponsive AMML cells but was found on the majority of responsive AMML cells; furthermore, 4 of the 5 patients with APL whose cells had lost the 1a-like antigen and thus showed some commitment to granulocytic differentiation, were unresponsive to TPA, whereas the one APL patient whose cells manifested the OKM1 antigen was responsive. Chromosome abnormalities characteristic of APL ([t(15;17)] (30) or found in AML but not AMML cells [t(8;21)] (31) were also associated with resistance to TPA.

The potential utility of TPA in autologous marrow reconstitution lies in the differential between the responsiveness of leukemic cells and the resistance of normal stem cells to the effects of TPA. Others have demonstrated preferential inhibition of leukemic CFU over normal GM-CFU in TPA concentrations >10^-9 M (32). In our studies, normal marrow incorporation of [3H]dThd, GM-CFU numbers, and normal GM-CFU proliferation into colonies was only mildly impaired by such treatment indicating that hematopoiesis is preserved following TPA exposure. In the two patients treated, repopulation of erythroid and myeloid precursors and of megakaryocytes occurred in a manner similar to that reported for autologous reconstitution with untreated marrow (33-35). Other parameters of the immune function (serum immunoglobulin levels, B-cell reactivity posttransplant, T-cell subset numbers, and ratio) also appeared to parallel those reported in autotransplanted patients; thus, TPA treatment of human bone marrow allows reconstitution and, presumably, preservation of the pluripotent stem cell.

We cannot be certain that there was no systemic effect of TPA that became unbound after infusion or that there would not have had the patients survived longer. Both autopsies failed to reveal any evidence of the intravascular aggregates of platelets and granulocytes seen in rabbits after TPA infusion (16), and no new lesions atypical for transplanted patients with acute leukemia were noted.

Since TPA is a tumor promoter and an inhibitor of induced maturation in certain cell lines (36), there remains a concern that its infusion increases the likelihood of leukemic cell proliferation. Including low concentrations of TPA in leukemic cell cultures has been reported to result in increased blast cell proliferation. Koeffler (37) has shown that the cloning efficiency of the myeloid cell line, KG-1, which requires colony stimulating activity for cell growth in agar culture, is increased by exposure to 5 x 10^-11-10^-9 M TPA. These concentrations did not affect normal marrow colony growth. Chang and McCulloch (38) also found that at concentrations of less than 1.6 x 10^-10 M, TPA enhanced the self-renewal of AML cells; however, in accord with our findings, TPA at concentrations >1.6 x 10^-9 M markedly decreased leukemic cell growth, and the cells present had macrophage characteristics. Although we exposed leukemic cells to TPA concentrations far in excess of 10^-9 M, it is theoretically possible that removing free TPA might change leukemic cell culture conditions to those of the lower concentrations of TPA which facilitate leukemic cell growth. Our experiments indicate that this does not occur. Cultures of leukemic cells showed a marked decrease in leu-
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kemic cell proliferation after short-term exposure to a high concentration of phorbol ester. Leukemia did return in both patients after reconstitution. Recurrence could have been due to residual leukemic cells not eradicated by the marrow-ablative preparative regimen or to reinfused cells resistant to the TPA effect. We were unable to document whether there were any residual leukemia cells in the reinfused marrow capable of proliferating, because marrow treated in this fashion is not suitable for standard long-term culture. The two treated patients were in a second and a third remission, respectively. Allogeneic marrow transplant in such patients is associated with a 28% relapse rate (39) suggesting the limited efficacy of ablative therapy in eliminating residual leukemic cells.

Under the more favorable conditions of first remission, allogeneic marrow transplant is limited to less than 10% of all adults with ANLL due to the need for a histocompatible donor and to the increased likelihood and severity of graft versus host disease with advancing patient age. Syngeneic bone marrow transplant is associated with a higher relapse rate than allogeneic transplant, presumably because of beneficial effects of graft versus leukemia in allogeneic transplant (40). This syngeneic experience is more analogous to the circumstances of autologous transplant, presumably because of beneficial effects of graft versus leukemia in an autologous transplant (40). This syngeneic experience is more analogous to the circumstances of autologous transplant, presumably because of beneficial effects of graft versus leukemia in an autologous transplant (40).

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