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Clinical Application of Phorbol Diester-induced Leukemic Cell Differentiation for the Definite Diagnosis of Acute Leukemias

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Seventy-three patients with acute leukemias or chronic myelogenous leukemias in blast crisis were evaluated as to the susceptibility of their leukemic cells to differentiation induction by a chemical agent, 12-O-tetradecanoyl phorbol-13-acetate (TPA). Leukemic cells of myeloid origin treated with TPA showed monocyte-macrophage-lineage differentiation morphologically and functionally, whereas those of lymphoid origin did not. We applied these differentiation phenomena for the clinical diagnosis of three leukemia cases in whom it was difficult to determine whether the leukemic cells were of non-lymphoid or lymphoid origin, although all the regular diagnostic procedures available had been performed. We successfully diagnosed these three cases by utilizing the above differentiation phenomena. Furthermore, this technique was clinically beneficial as to the choice of adequate chemotherapy in each of these leukemia cases. These findings confirm that the responsiveness to TPA of leukemic cells is of clinical usefulness for the definite diagnosis of acute leukemias.

Key word: Acute leukemia — Leukemic cell differentiation — 12-O-Tetradecanoyl phorbol 13-acetate

Human myelogenous leukemia cell line cells have the ability to differentiate into functionally mature cells when stimulated with various chemical inducers.[1] 12-O-Tetradecanoyl phorbol-13-acetate (TPA)*[4] is the most potent inducer of the terminal differentiation of human myelogenous leukemia cell lines into macrophage-like cells.[2-6] We previously reported, in a study on 26 cases of fresh leukemias, that myeloid leukemia cells cultured with TPA became adherent to plastic culture dishes, and then developed a macrophage-like morphology with long filamentous bipolar processes or projections within 48 hr and also showed marked enhancement of their phagocytosing ability.[7] On the other hand, lymphoid leukemia cells did not show such morphological or functional changes when cultured with TPA.[7]

The recently established French-American-British (FAB) classification scheme has been valuable for the diagnosis of acute leukemias.[8,9] However, even though diagnostic tests were performed carefully, we have sometimes experienced clinical difficulty in making a differential diagnosis between myeloid and lymphoid leukemias, and, consequently, in making a suitable choice of chemotherapeutic regimens.

In the present study, we investigated whether or not these characteristic changes could be applied on a greater scale for fresh leukemia cases, and furthermore, we discussed the application of these phenomena for a differential diagnosis in cases of fresh leukemias where it was difficult to determine whether the leukemic cells were of non-lymphoid or lymphoid origin.

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*[4] Abbreviations used: TPA, 12-O-tetradecanoyl phobol-13-acetate; AML, acute myeloblastic leukemia; APL, acute promyelocytic leukemia; ALL, acute lymphoblastic leukemia; AMMoL, acute myelomonocytic leukemia; AMoL, acute monocytic leukemia; CML-BC, chronic myelogenous leukemia in blast crisis; FAB classification, French-American-British classification.

Materials and Methods

Patient Population Seventy-three leukemia patients took part in this study. Seventy cases could be clearly diagnosed according to the FAB classification into 46 cases of acute myelogenous leukemias, subclassified as AML(M1), AML(M2), APL(M3), AMMoL(M4) and AMoL(M5), 13 of acute lymphoid leukemia (L1 and L2), and 11 of chronic myelogenous leukemia in blast crisis (CML-BC). In the other 3 cases it was difficult to determine the leukemia subclasses conclusively at the time of admission.

Preparation of Leukemia Cells Heparinized peripheral blood or a bone marrow aspirate was obtained from each patient at the time of admission. Mononuclear cells were isolated by the Ficoll-Hypaque gradient centrifugation technique. The cells were suspended in RPMI 1640 medium (Flow Lab., McLean, Virginia) supplemented with 10% heat-inactivated fetal calf serum (FCS; Commonwealth Serum Lab., Melbourne) in plastic culture dishes (No. 3003; Falcon, Oxnard, California), followed by incubation for 2 hr at 37° in a humidified atmosphere of 5% carbon dioxide. Then the non-adherent cells were collected, washed twice with FCS-containing RPMI 1640 medium and resuspended at the density of 2×10^5 cells/ml in culture dishes (No. 3002; Falcon). Ninety percent or more of these cells were leukemic, as judged by morphological assessment after staining with Wright-Giemsa solution, and the viability of the cells determined by the erythrosine B dye exclusion test was consistently 98% or more. The cells were incubated for 24 hr at 37° in a humidified atmosphere of 5% carbon dioxide. TPA (Sigma, St. Louis, Missouri) was added to the culture dishes to the final concentration of 16.2n*M*.

Morphological and Functional Analyses after TPA Induction After 24 hr incubation of the cultures, the morphology and cell adherence to plastic culture dishes were examined under an inverted microscope. Cells were gently scraped off a dish with a rubber policeman, and the viable cells were counted by means of erythrosine B dye exclusion. For morphological assessment of cells, cytospin smear preparations of the cells were prepared with a Shandon Cytospin centrifuge (Shandon Southern Products Ltd., UK) and stained with Wright-Giemsa solution. Specific and non-specific esterase double-stainings[10] were simultaneously performed on the cytospin preparations.

Phagocytic activity was determined by counting the number of cells that phagocytosed more than one yeast particle.[11]

Other Tests As diagnostic procedures, the following cytochemical stainings in addition to the regular Wright-Giemsa and esterase double stainings were performed: myloperoxidase, periodic acid-Schiff (PAS) and acid phosphatase. Terminal deoxynucleotidyl transferase (TdT) activity was assayed by the biochemical method.[12] Sheep erythrocytes were used for the E rosette-formation assay, and cell surface membrane immunoglobulins were investigated by using FITC-labeled rabbit anti-human immunoglobulin. Surface antigens re-

Table I. Cell Adherence and Phagocytic Activity Induced by TPA in Leukemic Cell Subtypes

Leukemia cell type (No. of cases)	Cell adherence (%)[a]		Phagocytic activity (%)[b]	
	Total adherent cells	Projection-forming cells	Control	TPA-treated
Acute leukemia				
Myeloid (46)	71.3±5.1[c] ┐	17.9±2.8 ┐	5.2±1.1 ┌─*─┐	22.3±2.6 ┐
Lymphoid (13)	0.2±2.2 ┘*	0.0±0.0 ┘*	1.0±0.4	2.2±0.5 ┘*
CML-BC				
Myeloid (5)	83.2±6.0 ┐	10.6±3.2 ┐	4.2±2.5 ┌─*─┐	21.8±4.7 ┐
Mixed (4)	29.9±9.2 *	0.7±0.2 *	3.0±1.5 ┌─*─┐	15.1±4.1 *
Lymphoid (2)	4.5±0.6 ┘	0.0±0.0 ┘	3.0±0.8	5.8±0.6 ┘

a) Cell adherence was assayed after 24 hr incubation of the cells with 16.2n*M* TPA. Control cultures showed no apparent cell adherence to plastic dishes.
b) Phagocytic activity in the control cultures before TPA treatment was compared with that in the TPA-treated cultures incubated for 24 hr.
c) Mean ± standard error of mean.
* $P<0.01$ (paired *t*-test).

active with established monoclonal antibodies, such as OKIa1, OKM1 (Ortho Pharmaceutical Corp., Raritan, New Jersey) and J5 (CALLA; Coulter Immunolgy, Hialeth, Florida), were observed by the indirect immunofluorescence method.

Results

Effect of TPA on Leukemia Cells Leukemic cells incubated with TPA for 24 hr in suspension cultures showed a slight decrease in cell number compared to the control cultures without TPA, and the cell viability in both the treated and control cultures was consistently greater than 85%.

The myeloid leukemia cells cultured with TPA became adherent to plastic culture dishes, and developed a macrophage-like morphology with long filamentous bipolar processes or projections within 24 hr (Table I, Fig. 1A, 1B and 1C). Cytochemical staining of preparations demonstrated that these cells treated with TPA were induced to differentiate along the monocyte-macrophage lineage. Furthermore, these myeloid leukemic cells cultured with TPA showed marked enhancement of their phagocytosing ability (Table I). There was no significant difference in the intensity of phagocytic activity or cell adherence induced by TPA among the myeloid leukemia subclasses (M1–M5, FAB

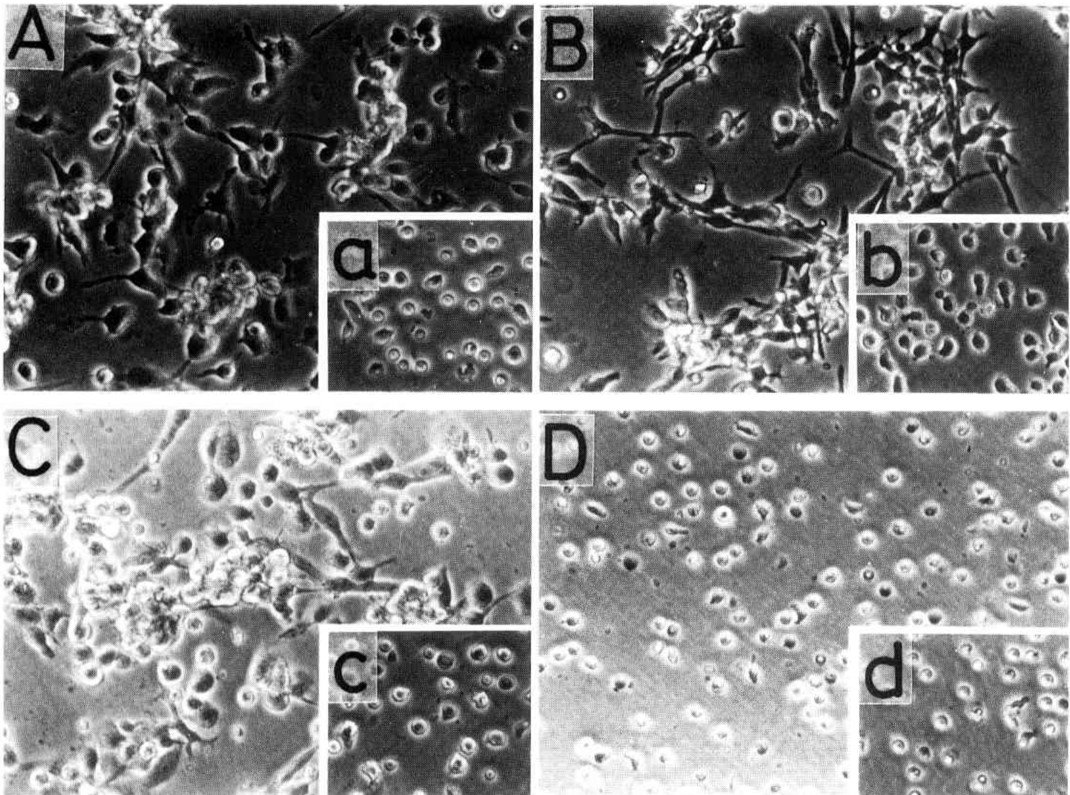


Fig. 1. Representative morphological changes of leukemic cells treated with TPA (inverted microscopic observation, ×200). Each photo shows the morphology after incubation with 16.2n*M* TPA for 24 hr. A, a case of acute myelomonocytic leukemia (M4); B, a case of acute myeloblastic leukemia (M1); C, a case of acute myeloblastic leukemia (M2); D, a case of acute lymphoblastic leukemia (L1). The lower right photo in each case (a, b, c and d) shows the control culture without TPA. Marked macrophage-like morphological changes with long filamentous bipolar processes or projections were observed in each myeloid leukemia case.

classification), and properties, such as cell adherence, the formation of bipolar processes or projections and phagocytic activity, did not always parallel each other.

The lymphoid leukemia cells cultured with or without TPA showed neither significant morphological changes nor phagocytic activity enhancement (Table I and Fig. 1D). In some cases, cell aggregates were observed with the TPA-treated lymphoid leukemia cells. However, in one exceptional case of acute lymphoblastic leukemia, leukemic cells showed adherence to plastic culture dishes, but there was neither projection-formation nor phagocytic activity.

The above-mentioned characteristic changes were also observed in leukemic cells from CML patients in blast crisis (Table I). In the mixed type of crisis, in which the blast cells consist of both myeloid and lymphoid cells morphologically, the appearance of both projections and cell aggregates was observed. The percentage of these cells showing phagocytic activity was intermediate between the values obtained for the myeloid and lymphoid crises of CML (Table I).

Studies on the Differentiation Phenomena in Three Leukemia Cases Where It Was Difficult to Determine the Leukemic Cell Lineages

Case 1 (J.T.) A 25-year-old women, complaining of general fatigue and a pale face, was admitted to our hospital. On admission, her peripheral white blood cell count was 79,400/mm^3, and 97.2% of the cells were blast cells. A bone marrow aspirate also showed marked hypercellularity with 95.8% of blast cells. We first diagnosed this case as AMoL(M5) morphologically on the basis of the FAB classification. Histochemically, about 3% of the blast cells were found to be weakly peroxidase-positive, PAS-negative and weakly alpha-naphthyl butyrate esterase-positive. Because of the hyperleukocytic stage of leukemia, an immediate treatment was needed for this case. We applied a chemotherapeutic regimen including BH-AC (N^4-behenoyl-1-β-D-arabinofuranosylcytosine, an N^4-acyl derivative of cytosine arabinoside),[13] aclacinomycin (ACM, a newly synthesized anthracycline antibiotic),[14] 6-mercaptopurine (6-MP) and prednisolone (PSL). In the meantime, we demonstrated that the leukemic cells

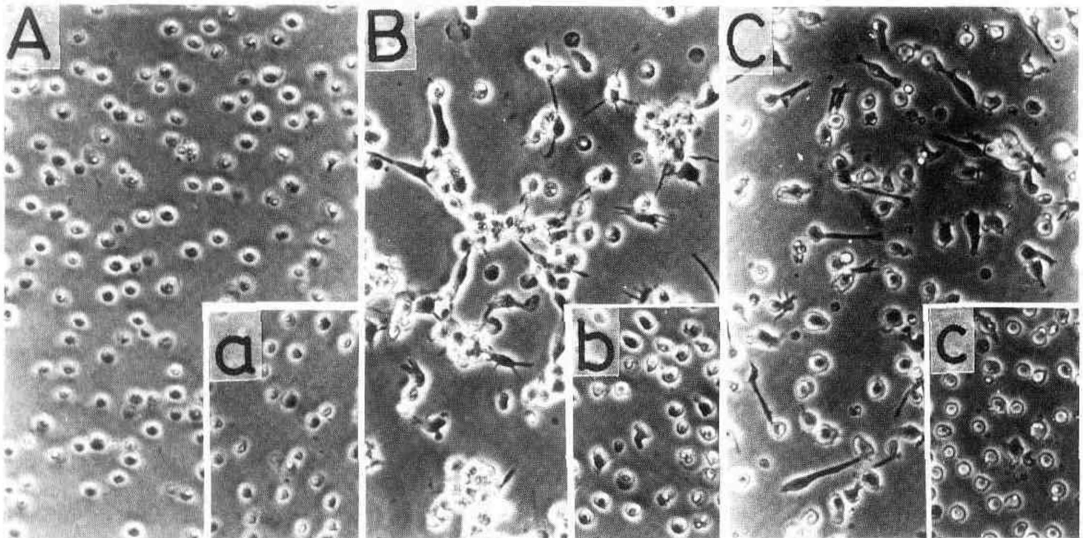


Fig. 2. Morphological changes of leukemic cells from three acute leukemia cases in which the clinical diagnosis was difficult to make (inverted microscopic observation, ×200). A, case 1; B, case 2; C, case 3. Leukemic cells were treated with 16.2n*M* TPA for 24 hr. The lower right photo in each case (a, b and c) shows the control culture without TPA. In cases 2 and 3, marked macrophage-like morphological changes with long filamentous bipolar processes or projections were observed.

in bone marrow, obtained from this patient at the time of admission, showed neither cell adherency to plastic culture dishes nor acquisition of phagocytic activity after treatment with TPA in culture (Fig. 2A, 3A and 3B). In Wright-Giemsa-stained preparations, the TPA-treated cells showed no morphological changes when compared with non-treated cells. When cytochemical and biochemical examinations were performed again in this case, the results were as follows: alpha-naphthyl butyrate esterase, for which the leukemic cells were weakly positive, was not inhibited by sodium fluoride. Surface antigens that reacted with established monoclonal antibodies such as OKIa1 and J5(CALLA) were found to be present in the leukemic cells according to the indirect immunofluorescence method, and 93.0% of the leukemic cells were TdT activity-positive. Finally, we changed the diagnosis in this case from AMoL to ALL(L2), and we obtained complete remission in this case by applying a chemotherapeutic regimen including L-asparaginase (L-asp), daunorubicin (DNR), vincristine (VCR), BH-AC and PSL, which has been widely used for the treatment of adult ALL[15] (Fig. 4).

Case 2 (F.K.) A 51-year-old man, complaining of fever, was admitted to our hospital. On admission, his peripheral white blood cell count was 22,000/mm^3 with 62.5% of blast cells. A bone marrow aspirate showed moderate cellularity with 95.4% of blast cells. We diagnosed this case as ALL(L2) morphologically according to the FAB classification. On histochemical examination, about 3% of the leukemic cells were found to be weakly peroxidase-positive and weakly alpha-naphthyl butyrate esterase-positive. We demonstrated that these leukemic cells in the culture with TPA changed to adherent cells with long filamentous projections, acquired phagocytic activity (Fig. 2B, 3C and 3D), and showed a macrophage-like morphology on Wright-Giemsa staining. Further biochemical examinations revealed that these leukemic cells were

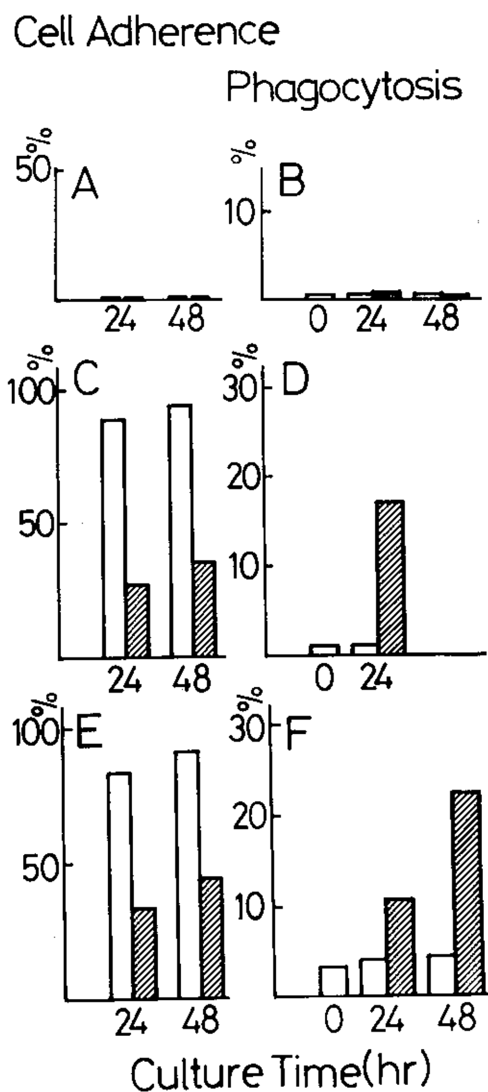
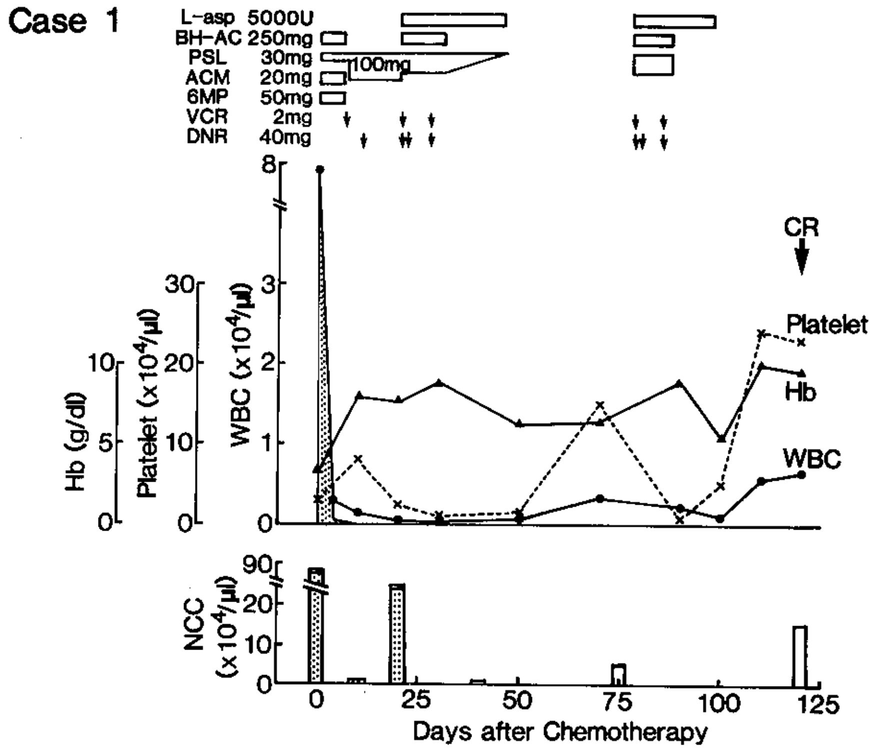


Fig. 3. Effects of TPA on cell adherence and phagocytic activity in three leukemia cases. A, B, case 1; C, D, case 2; E, F, case 3. A, C and E, the effect of TPA on cell adherence in each case. The empty columns show the percentages of total adherent cells cultured with TPA for 24 and 48 hr. The shaded columns show those of long filamentous projection-forming cells. The control culture without TPA in each case did not show any cell adherence to the culture dish bottom. B, D and F, the effect of TPA on phagocytic activity in each case. The shaded columns show the percentages of phagocytosing cells after culture with TPA for 24 and 48 hr. The empty columns show those of cells cultured without TPA. In cases 2 and 3, marked enhancement of phagocytic activity was observed after TPA treatment.



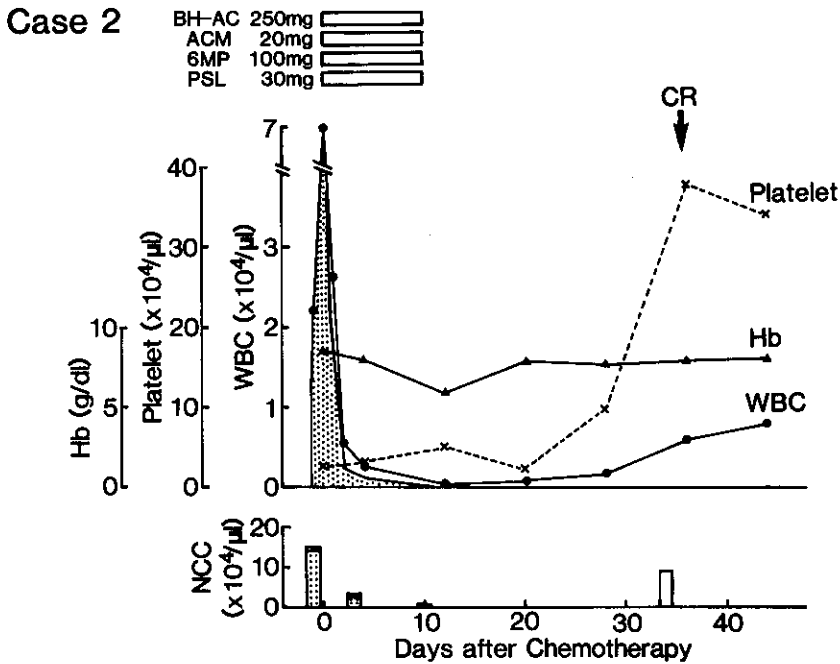


Fig. 4. The time courses of clinical responses to the successful chemotherapeutic regimens administered after changes of clinical diagnosis on the basis of differentiation-induction by TPA. The shaded area represents blast cells. Abbreviations used are as follows: CR, complete remission; NCC, nuclear cell count. Other abbreviations were described in the text.

positive as to surface antigens that reacted with monoclonal antibodies such as OKIa1 and OKM1, and negative as to J5(CALLA). Finally, we changed the diagnosis in this case from ALL to AML(M1), and applied a chemotherapeutic regimen including BH-AC, ACM, 6-MP and PSL, which has been reported to be an effective treatment for acute myelogenous leukemias.[16] We obtained complete remission in this case after this treatment, as shown in Fig. 4.

Case 3 (M.E.) An 18-year-old man, complaining of fever, general fatigue and retinal bleeding, was admitted. On admission, his peripheral white blood cell count was 107,000/mm^3 with 95.0% of blast cells, of which about 3% were peroxidase-positive. We first diagnosed this case as ALL(L2) morphologically according to the FAB classification. Further studies demonstrated that these leukemic cells in culture changed to adherent cells with long filamentous processes and acquired phagocytic activity (Fig. 2C, 3E and 3F), when treated with TPA. Cytochemical and biochemical examinations were performed again, and revealed that the weak activity of alpha-naphthyl butyrate esterase in leukemic cells was completely inhibited by sodium fluoride, and that the leukemic cells were negative as to E-rosette formation and had no surface immunoglobulins. TdT activity was absent. We finally changed the diagnosis in this case from ALL to AMoL (M5), and instead of the initial ALL therapy combined with VCR and PSL, we applied a regimen including cytosine arabinoside, DNR, 6-MP and PSL, which has also been reported to be an effective regimen for acute myelogenous leukemias.[17] Marked cytoreduction was observed, but the patient died of a cerebral hemorrhage due to thrombocytopenia.

Discussion

We have presented evidence of the usefulness for the clinical diagnosis of fresh acute leukemias of observing the susceptibility of leukemic cells to monocyte-macrophage lineage differentiation induced by TPA, as determined in a study on 73 patients with fresh leukemias. The results of this large-scale study confirmed our previous finding that TPA-treated myeloid leukemic cells became adherent to the substratum, developed a macrophage-like morphology with long filamentous bipolar processes or projections, and acquired phagocytic activity.[7] It is well recognized that myeloid leukemia cells have the capacity to differentiate along a monocyte-macrophage lineage in response to stimulation with TPA.[18-20] In addition, some acute undifferentiated leukemia (AUL) cells also exhibited the monocytic differentiation phenomenon when induced with TPA.[21] In our present study, we showed that the TPA-induced differentiation occurred within a shorter incubation period when compared with that observed in other studies. This indicates its usefulness for making a differential diagnosis as to whether leukemic blasts are of non-lymphoid or lymphoid origin in a shorter time after admission for each leukemia patient, and consequently for making a suitable choice of chemotherapeutic regimens for acute leukemias.

We should emphasize that two patients, cases 2 and 3, at first diagnosed as having ALL according to the FAB classification, were finally diagnosed as having acute myelogenous leukemia, and one patient, case 1, was finally determined to have ALL instead of AML, on the basis of the cell-differentiation phenomena in addition to precise cytochemical observations, i.e. cell sorter analyses using established monoclonal antibodies such as OKIa1, OKM1, OKM5 and J5, and some hematologically specific stainings for detecting enzymatic activities of myeloperoxidase, acid and alkaline phosphatases, specific and non-specific esterases, and PAS-positivity. Furthermore, in cases 1 and 2, complete remission was obtained with suitable chemotherapy after diagnostic reevaluation of the leukemic cell origin. Case 3, which we reported on partially elsewhere,[7] also responded to the chemotherapy selected after reevaluation of the cell origin of his leukemic blasts.

It has also been reported that some lymphoid leukemia or hairy cell leukemia cells show cell adherence or projection-formation in response to TPA,[22,23] and some lymphoid leukemia cells showed lymphoid differentiation phenotypically induced by TPA.[24-26] In the present study, leukemic blasts from one case with ALL showed cell adherency to the substratum but did not exhibit either pro-

jection-formation, morphological changes or phagocytic activity. A more precise investigation is now in progress.

Our conclusion is that observation of the response to exposure of leukemic cells to TPA for a short time might be valuable for evaluating their cell lineage, i.e. lymphoid or non-lymphoid, and this might allow a quicker and better choice of chemotherapies for acute leukemias. Further studies must be performed to clarify the possible mechanisms of cell differentiation-induction by various chemical inducers and biological response modifiers, and to apply such differentiation-induction to the therapy of acute leukemias.

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