Terminal Transferase Positive Acute Promyelocytic Leukemia: In Vitro Differentiation of a T-Lymphocytic/Promyelocytic Hybrid Phenotype

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In a case of acute promyelocytic leukemia (APL), the expression of terminal deoxynucleotidyl transferase (TdT), an early lymphoid marker, was detected. Double-fluorescent staining for the myeloid-specific antigens VIM-2 and VIM-D5 in combination with specific antiserum for TdT suggested a mixed leukemic cell population consisting of a morphologically, cytochemically, and immunologically promyelocytic component (80%) and a lymphoid, TdT+ component (20%) that was myelomonocytic in morphology but otherwise without any evidence of nonlymphoid nature. Fluorescent-activated cell analysis revealed that a greater number of cells reacted with monoclonal anti-T antibodies (OKT3, OKT6, and OKT11) than could be identified as lymphoid by TdT expression. As confirmed by double-staining fluorescence microscopy, a large fraction of the promyelocytic leukemia cells were biphenotypic, expressing both myeloid and lymphoid markers (50% positive for VIM-D5 and OKT6, 30% positive for VIM-D5 and OKT3). Subsequently, in vitro differentiation experiments were performed. While treatment of the cells with GCT-conditioned medium favored proliferation, with only a weak and delayed promotion of the cells towards maturation as reflected by enhanced expression of the mature T-marker T3 but persistent expression of the thymocyte antigen, exposure to all-trans and 13-cis retinoic acid resulted in marked differentiation of both the myeloid and the lymphoid cell characteristics. Retinoic treatment resulted in the loss of TdT, a partial disappearance of the T6-antigen, and the expression of the late T cell antigen T3 by almost 70% of the cells. In addition, myeloid maturation was obvious from the morphologic appearance of the cells, as well as from the expression of the OKM1-associated antigen by a majority of the cells. This report concerns a unique case of APL in which, for the first time, a coexistence of promyelocytic and lymphoid elements was detected, with exposure of the cultured leukemic cells to retinoic acid inducing maturation along both the myeloid and the lymphoid lineage.

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That phenotypic heterogeneity may be present in individual leukemia cell populations has become obvious since the addition of refined immunologic and enzymatic markers for classification to conventional morphologic and cytochemical criteria. Rare cases of leukemia have been reported in which the malignant population was found either to be composed of distinct, antigenically different subtypes of leukemic cells, or to contain a varying portion of cell hybrids expressing both myeloid and lymphoid characteristics. In many instances, such diversity has been indicated by increased activity of the enzyme terminal deoxynucleotidyl transferase (TdT), an accepted marker for lymphoid precursor cells, in otherwise typical nonlymphoid leukemias. Because of the nuclear distribution of the TdT enzyme and the surface expression of myeloid differentiation antigens, double staining using specific TdT antiserum and myeloid-specific monoclonal antibodies is possible, allowing precise identification of a cell as “mixed” or “biphenotypic.”

In addition to raising the question of mono- or polyclonality, double-marker leukemias have attracted the special interest of clinical hematologists, since they have an unfavorable prognosis. To date, neither chemotherapeutic regimens designed for acute myeloid or lymphoid malignancies alone nor a combination of both has proved to be of long-term benefit to these patients. A new approach to therapy therefore would be highly desirable.

Sachs and coworkers have introduced the concept that leukemic transformation is caused by the uncoupling of control mechanisms for growth and differentiation during normal hematopoiesis. Deficient maturation could result from an altered or impaired ability of hematopoietic precursor cells to respond to normal, differentiation-inducing factors. There are abundant preliminary data that suggest maturation arrest need not be irreversible, since a number of biologic response modifiers as well as nonphysiologic agents are able to induce leukemic cells to overcome the differentiation block, at least in vitro. Increasingly frequent reports on the successful induction of differentiation in patients with myeloid leukemia, however, give promising evidence for the applicability of the in vitro experience to the treatment of the disease. In contrast to the data available on myeloid cells, reported attempts to induce differentiation of lymphoid lei-

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kemic cells are few and, thus far, have been restricted to cell cultures. Since compounds found to be the most effective in propagating differentiation of lymphoid cells are highly inflammatory, tumor-promoting phorbol esters, in vivo studies have not been performed. With regard to leukemias having both myeloid and lymphoid features, induction of differentiation has not been reported.

MATERIALS AND METHODS

Culture Conditions

During initial evaluation of patient S.K., bone marrow was aspirated into a heparinized syringe. Mononuclear cells were separated from this specimen by Ficoll-Hypaque gradient centrifugation. The isolated blast cells were washed, suspended in the high glucose concentration (4.5 g/L) formulation of Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY), supplemented with 10% fetal calf serum (Flow Laboratories, McLean, VA), 100 μg/mL streptomycin, 100 U/mL penicillin, and cultured in plastic tissue culture flasks (Falcon, Becton Dickinson, Oxnard, Calif) at 37°C in a humidified atmosphere of 7.5% CO2 in air. Growth was constantly observed by an inverted microscope (Nikon, Garden City, NY). After the cell culture was exhausted and fresh leukemic material was not available because the patient remained in complete remission, some additional marker studies were performed on cells that had been stored in liquid nitrogen.

In Vitro Induction of Differentiation

For differentiation studies, floating, nonadherent cells were harvested and subcultured at 5 x 10⁶ cells per milliliter in culture medium containing either 30% GCT-conditioned medium (GIBCO), 1-10 x 10⁻⁴ mol/L all-trans retinoic acid (Sigma Chemical Co, St Louis) or 13-cis retinoic acid (generously provided by Hoffmann-La-Roche, Nutley, NJ) dissolved in absolute ethanol (final ethanol concentration in the culture medium was 0.1%), or 0.1% ethanol to serve as a control to the retinoic acid experiments. All procedures involving retinoids were done in subdued light, and incubations were performed at 37°C. The isolated blast cells were washed, suspended in the high glucose concentration (4.5 g/L) formulation of Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY), supplemented with 10% fetal calf serum (Flow Laboratories, McLean, VA), 100 μg/mL streptomycin, 100 U/mL penicillin, and cultured in plastic tissue culture flasks (Falcon, Becton Dickinson, Oxnard, Calif) at 37°C in a humidified atmosphere of 7.5% CO2 in air. Growth was constantly observed by an inverted microscope (Nikon, Garden City, NY). After the cell culture was exhausted and fresh leukemic material was not available because the patient remained in complete remission, some additional marker studies were performed on cells that had been stored in liquid nitrogen.

Routine Cytologic Examination

Smears from the harvested cells were prepared by cytocentrifugation (Shandon Southern Products, Sewickley, Pa). Morphologic evaluation after Wright-Giemsa staining was supplemented by histochemical staining for myeloperoxidase and butyrate esterase.

Monoclonal Antibodies

The monoclonal VIM-2 and VIM-D5 hybridoma antibodies were generously supplied by Dr W. Knapp, Institute of Immunology, Vienna, Austria. VIM-2 reacts specifically with all myeloid cells including myeloblasts. VIM-D5 reacts with a differentiation antigen expressed at and above the level of promyelocytes. OKM1, purchased from Ortho Laboratories (Raritan, NJ), only detects relatively mature myeloid cells, leaving myeloblasts and promyelocytes undetected.

The monocyte-specific monoclonal antibody anti-human Monocyte 1 was obtained from Bethesda Research Laboratories (Bethesda, Md).

The monoclonal antibodies OKT3, OKT6, and OKT11 were kindly provided by Dr A. Rubenstein, Albert Einstein College of Medicine, Bronx, NY. OKT3 is exclusively reactive with T cell antigen present normally on mature thymocytes and peripheral blood lymphocytes. OKT6 is the thymic differentiation antigen. The OKT11-associated antigen identifies the sheep erythrocyte receptor. The monoclonal antibody developed against the common acute lymphocytic leukemia antigen (CALLA) was made available to us by Dr Knapp.

Binding of the antibodies to the leukemic cells was generally evaluated by indirect immunofluorescence with fluoresceinated, affinity-purified F(ab')2 fragment goat anti-mouse immunoglobulins (Cappel Laboratories, Cochranville, Pa), and in the case of the VIM-D5 antigen by direct immunofluorescence with tetraethylrhodamine isothiocyanate-labeled VIM-D5 hybridoma antibody.

Fluorescent OKT3, OKT6, and OKT11 antibody-coated cells were detected on a fluorescence-activated cell analyzer (FACS-1, Becton Dickinson, Sunnyvale, Calif). Background fluorescence was determined by staining cells with a control IgG antibody, and the percentage of cells more fluorescent than the background was recorded. For T6 and T3, staining was also demonstrated by fluorescence microscopy. Fluorescence intensity of cells carrying T antigens was comparable with that of myeloid-staining cells.

Terminal Deoxynucleotidyl Transferase (TdT) Assays

Assays of TdT were accomplished by biochemical determination using the micromethod developed by Modak et al and by immunological determination. For the latter, indirect immunofluorescent staining was performed on cytosin smears (approximately 5 x 10⁶ cells per spot) after fixation in absolute methanol (30 minutes, 4°C) by incubating the cells with anti-transferase followed by fluorescein-conjugated F(ab')2 goat anti-rabbit IgG for 30 minutes at 30°C. Both the first and the second antibody were generously supplied by Dr F.J. Bollum, Uniformed Services University of the Health Sciences, School of Medicine, Bethesda, Md.

Double-fluorescence Staining

Double-staining was performed as previously described. In the case of VIM-2/TdT, the leukemic cells were first incubated in suspension with the VIM-2 hybridoma antibody, counterstained with fluorescein-conjugated second antibody (both for 30 minutes, 4°C), then distributed on glass slides by cyotspin centrifugation and processed for TdT determination as described before, with the exception that the second antibody for TdT was rhodamine-labeled F(ab')2 goat anti-rabbit IgG (Cappel Laboratories). In the case of VIM-D5/TdT, the cells were first incubated with the rhodamine-labeled isolated VIM-D5 hybridoma antibody followed by TdT determination as described above. Double-staining for myeloid and T cell antigens (VIM-D5/OKT6,OKT3) was achieved by labeling the cells first with the myeloid monoclonal antibody, counterstaining with rhodamine-conjugated IgG fraction goat anti-mouse IgG (Cappel Laboratories), followed by incubation with OKT6 or OKT3 and counterstaining with fluorescein-conjugated second antibody. Fluorescence of the cells was evaluated using a Nikon microscope with incident illumination and equipped for the dual wave-length method.

3H-thymidine (3H-TdR) Incorporation

Nonadherent mononuclear cells were harvested from the original tissue culture flask, checked for viability by trypsin blue exclusion, and distributed in microtiter plates (Falcon) in 0.1 mL aliquots (5 x 10⁶ cells/mL) suspended in culture medium containing either 30% GCT-conditioned medium, 1-10 x 10⁻⁴ mol/L all-trans or 13-cis retinoic acid, or 0.1% ethanol to serve as a control for the retinoic acid. After 4, 36, 60, 96, and 192 hours of culture, the rate of
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\(^{3}\)H-TdR uptake was evaluated by pulsing with 0.1 \(\mu\)Ci of \(^{3}\)H-TdR per culture (specific activity 40 Ci/mmol; Radiochemical Centre Amersham, Arlington Heights, Ill) for four hours before termination of the culture by harvesting on filter paper disks (mash II glass fiber filters, M.A. Bioproducts, Walkersville, Md) using the Skatron multisample harvesting apparatus (Skatron, Inc, Sterling, Va). The filters were washed with 5% trichloroacetic acid, rinsed with distilled water and absolute methanol, and counted for radioactivity (Tri-Carb Packard Scintillation Counter) using Aquasol (New England Nuclear, Boston) as the scintillation liquid.

Results of \(^{3}\)H-TdR incorporation under conditions of in vitro differentiation were expressed as the percentage of label incorporated by the cells under control conditions.

Cytogenetic Analysis

The methotrexate cell-synchronization technique of Yunis et al\(^{37}\) was used for karyotyping.

CASE REPORT

S.K. is a 59-year-old woman who, in August 1983, was examined for diffuse ecchymotic lesions. She was anemic (hematocrit, 16%) and thrombocytopenic (platelet count, 60,000/\(\mu\)L). Coagulation studies showed hypofibrinogenemia (fibrinogen, 80 mg/100 ml) and the presence of fibrin split products (1:80). Together with the finding of 30% hypergranulated promyelocytic blast cells in peripheral blood and 80% in bone marrow, the clinical picture was consistent with a diagnosis of acute promyelocytic leukemia (APL). The patient was treated with low-dose heparin and antileukemic therapy, which reversed the coagulopathy without significant bleeding and led to complete hematologic remission (more than 6 months).

RESULTS

Morphologically, the blast cell population isolated from the patient’s bone marrow was characterized as 80% promyelocytic (M3) and 20% myelomonocytic (M4) (Fig 1). Only the promyelocytic cell type stained with myeloperoxidase; that the entire cell population was negative for butyrate esterase was taken as evidence against a myelomonocytic nature of the nonpromyelocytic component. Likewise, the lack of reactivity with the monocyte-specific monoclonal antibody anti-human Monocyte\(_1\) was inconsistent with the morphologic picture. Biochemical determination of TdT activity revealed an elevated enzyme level of 0.44 U/10\(^6\) cells (normal level, 0.1 U/10\(^6\) mononuclear bone marrow cells), and 20% of cells stained with specific TdT-antiserum (15% strongly positive, 5% weakly positive). An association of TdT expression with the nonpromyelocytic cell type became likely when double-fluorescence staining with TdT-antiserum in combination with myeloid-specific monoclonal antibodies (VIM-2 and VIM-D5 hybridoma antibody) showed that lymphoid and myeloid markers were present on distinct cell populations (Fig 2).

In order to further characterize the lymphoid cell type obviously present side by side with the major promyelocytic cell line, staining for CALLA and the T-associated antigens T3, T6, and T11 was performed. The absence of CALLA as assessed from the lack of reaction with the VIL-A1 monoclonal antibody was inconsistent with pre-B characteristics. The T phenotype of the lymphoid cell component was confirmed when cell analyzing of OKT3, OKT6, and OKT11 coated cells revealed that 31%, 62%, and 45% of the cells stained with these T antigen-reactive monoclonal antibodies, respectively. The expression of the T6 antigen especially, together with the TdT data, classified the lymphoid phenotype as early T or pre-thymic T phenotype. The fact that the percentage of T-antigen–positive cells exceeded that of TdT\(^{+}\) cells, ie, nonpromyelocytic cells, indicated that at least a portion of the promyelocytic blasts carried T cell markers. To confirm this assumption, double-staining on the fluorescence microscope of the leukemic blasts with VIM-D5 hybridoma antibody and OKT6 as well as OKT3 was performed. As a result, approximately 50% of the myeloid-staining cells also carried the thymocyte antigen, and the T3-antigen was consistently present on VIM-D5 positive cells.

Shortly after the start of liquid culture of the bone marrow cells, fibroblast-like cells together with large adherent cells became apparent in one of the tissue culture flasks inoculated and only in this flask was growth of non-adherent cells found to continue. Within two weeks, the stromal monolayer was confluent and the floating mononuclear cells seemed to proliferate, preferentially attached to or around the centers of
large fibroblast patches consisting of giant, possibly fat, cells (Fig 3).

Cytogenetic analyses performed at diagnosis, after two days, and again after two weeks in culture showed that greater than 90% of the more than 70 mitoses examined were of normal karyotype (46XX) and in less than 10% a trisomy 8 was present (47XX,+8).

Attempts to induce differentiation of this leukemic cell population involved culturing cells with 30% GCT-conditioned medium or with 1-10 × 10^-6 mol/L all-trans and 13-cis retinoic acid for six to nine days. Table 1 summarizes the effects of these agents on the phenotype of the original cell population. After nine days of culture in the presence of conditioned medium, the morphologic picture was dominated by large, hypergranulated promyelocytes (Fig 4) that stained strongly for myeloperoxidase and showed no evidence of myeloid maturation. No TdT activity could be detected in this cell population. The increasing percentage of cells expressing the T-markers T11 and T3 indicated that, to some degree, differentiation along the lymphoid pathway had been initiated, even though the percentage of cells positive for T6 remained unchanged compared with the control culture. On the other hand, exposure of the cells to retinoic acid resulted in the loss of TdT accompanied by a partial disappearance of T6-antigen and a marked increase in the percentage of T3-positive cells. At the same time, morphologic and immunologic maturation along the myeloid pathway was observed. The hematologic picture after retinoic acid induction resembled that of chronic myelogenous leukemia with all differentiation stages of the myeloid cell lineage present, and a positive reaction of greater than 60% of the cells with the monoclonal antibody OKM1. The retinoic acid-treated cells had totally lost granulation but continued to contain nucleoli (Fig 5).

The difference between the effect of GCT-conditioned medium and that of retinoids was further elaborated by determining the rate of 3H-TdR incorporation as a measure of the proliferative activity of the cells. When S.K. cells were removed from the feederlayer culture to serve as a control in this experiment, the amount of label incorporated remained relatively constant during the total culture period of nine days (111 ± 6 cpm/10^6 cells) except for a transient rise within the first 36 hours. As expected from the marked differentiating effect of retinoic acid, 3H-TdR uptake was decreased to 50% of the control level (59 ± 2 cpm/10^6 cells, P < .001) within four days. At this time, cellular features were still those of control cultures; only during the following five days did phenotypic changes resembling maturation occur. In contrast, in GCT-conditioned medium-supplemented culture, 3H-TdR incorporation was almost tenfold higher than the control value after 36 hours (2,380 ± 116 cpm/10^6 cells) and continued to rise up to 50-fold greater than the control within 60 hours (4,500 ± 140 cpm/10^6 cells), but had dropped by day 9 (1,040 ± 70 cpm/10^6 cells). It should be emphasized that the viability of the cells during the time period tested was always greater than 90%. These data clearly show that GCT-conditioned medium favors proliferation of S.K. cells over differentiation. As with retinoic acid, the cells in GCT-conditioned medium required exposure to the inducer for at least six days before showing the phenotypic changes listed in Table 1.

**DISCUSSION**

The clinical and hematologic picture in patient S.K., which is characterized by the proliferation of morphologically abnormal promyelocytes and a severe bleeding diathesis, was consistent with the diagnosis of APL. The t(15;17) translocation, a structural chromosome rearrangement found to be nonrandomly associated with APL, could not be detected, despite the use of the methotrexate cell-synchronization technique. In-
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stead, greater than 90% of the mitoses examined showed a normal karyotype, and in less than 10% was a trisomy 8 (47XX,+8) present. Besides being the most frequent aberration seen in acute nonlymphocytic leukemia (ANLL), a gain of No. 8 has been found to occur frequently in addition to the t(15;17) anomaly, and occasionally to be the only chromosomal change in APL.

Our patient is the first reported case of APL in whom elevated levels of TdT were detected at a percentage exceeding by far that of TdT- prothymocytes normally present in the bone marrow (up to 5%). As shown by double-fluorescent staining, these TdT- blasts were clearly distinct from the more numerous promyelocytic cells. Positivity of a large portion of the promyelocytic (V1M-D5 positive) cell population for T-antigens in double-staining microscopy as well as FACS-analyzing suggested, however, the additional presence of a leukemic hybrid-phenotype. Previously, the unexpected participation of lymphoid components has been documented in blast crises of chronic myelogenous leukemia and in acute leukemias classified as types M1, M2, or M4 by the morphologic criteria detailed in the French-American-British (FAB) classification. The only indication of a possible lymphoid involvement in APL (M3) so far has been a rare report of meningeal relapses, a complication most frequently occurring in acute lymphocytic leukemia.

In order to explain the occurrence of biphenotypic leukemias, the involvement of a common myeloid/lymphoid committed precursor cell or the aberrant reexpression of genomes coding for markers of the other cell lineage during leukemic transformation could be invoked. In any case, the fact that in most such patients only part of the cells show the double marker expression raises the question whether these cells and the residual leukemic population originate from the same stem cell or represent separate leukemic entities blocked at different stages of maturation. As described in a case by Griffin et al., one potential method for answering this question is the screening for such specific chromosomal aberrations as the Philadelphia chromosome, which was found to be present in both the hybrid phenotype and the remaining cell population of their patient with CML-BC. In our patient, follow-up of phenotypic alterations occurring during induction of differentiation in vitro gave strong evidence for the monoclonality of the leukemic blast population. The propagation of maturation revealed that the various phenotypes characterized initially had represented various stages of differentiation of one and the same precursor cell committed to develop in a unique way from a prethymocytic (positive for very early, unidentified myeloid markers?), morphologically myeloid stage through a lymphoid/promyelocytic stage to a pure promyelocytic stage. This was clearly reflected by the decrease in the initially predominant expression of the T6-antigen on the promyelocytic blasts and the increased expression of the late T cell antigen T3 associated with RA-propagated maturation. It was interesting to note that despite definite signs of myeloid maturation under RA, nucleoli were consistently present, which confirmed that these myelocytes, metamyelocytes, and polymorphonuclear leukocytes derived from the original leukemic cells. This is in clear distinction to the "normal" cells characterizing remission following chemotherapy.

Supplementation of S.K. cultures with medium conditioned by the human monocytoid cell line GCT, a potent source of colony-stimulating activity (CSA) for normal human marrow, sustained and strongly enhanced the proliferative activity of the cells. After nine days of exposure to GCT-conditioned medium, cell proliferation decreased despite greater than 90% viability of the cells. Even though at this point the cells still showed hypergranulated, promyelocytic appearance, the decrease in the number of TdT- cells and the enhanced expression of T3 on the majority of the cells points toward a primary selectivity of the maturation process for the lymphoid component of the cell population. Using 30% activated T lymphocyte-conditioned medium, Chiao et al. demonstrated the rapid onset of differentiation in leukemic null-lymphocytes, but also in this case, increasing proliferation of the imma-
tured cells preceded differentiation. In the case of S.K., the large and prolonged proliferation–stimulatory effect of GCT-conditioned medium is in line with the total dependence of in vitro growth of the cells on a feederlayer of giant, fibroblast-like cells. When seen in normal bone marrow cultures, these adipocyte-containing confluent stromal layers have been found to be the source of CSA for the nonadherent mononuclear cell population.43 Interestingly, in another case of myeloid leukemia recently reported by Sato et al44 to be dependent on fibroblast-like, adherent cells, an extra chromosome 8 was the only abnormality present at all occasions.

With regard to the induction of differentiation in S.K. cells by vitamin-A analogues, all-trans retinoic acid and 13-cis retinoic acid were equally effective, as was also reported for the retinoic acid-mediated inhibition of the clonal growth of human myeloid leukemia cells, including the HL-60 line.45 The effectiveness of retinoic acid in inducing differentiation has long been thought to be restricted to the promyelocytic type of leukemia, but evidence for its effectiveness in other types of acute nonlymphocytic leukemia has recently been provided as well.46 With respect to a differentiating effect of retinoic acid on leukemic cells of the lymphoid type, however, previous reports in the literature have been quite discouraging. In T-ALL cell lines, retinoic acid did not modulate the immature phenotype as assessed by the persistent expression of TdT activity.22,23,25 The compounds found to be effective in increasing the E rosetting capability of leukemia lymphocytes, in decreasing TdT activity, or in changing the cell surface antigen pattern to that of a more mature state are dimethylsulfoxide and phorbolester.22–29 With regard to the action of retinoic acid on normal lymphoid cells, a stimulation of the response of human blood lymphocytes to mitogens or antigens has been found in some studies,47 and in others it has been found to be an inhibition.48 Augmentation of the proliferative response would suggest promotion of maturation of appropriate lymphoid precursor cells, especially in view of data showing that during normal T cell development in rodents, the decrease in TdT+ cortical thymocytes is associated with large increases in the mitogenic responsiveness of the cells.49 The lack of evidence for maturation induction by retinoids in the lymphoid leukemic blast cells tested so far might be attributable to the fact that all of these cell lines are highly selected clones representing cells blocked at specific stages of differentiation,50 with the level of maturation block determining the ultimate response of the leukemic target cell to inducing substances. Even with 12-O-tetradecanoyl-phorbol 13-acetate (TPA), the effectiveness as differentiation inducer has been found to vary with the type of lymphoid leukemic cell line tested.22 Furthermore, as pointed out by Sachs,14 induction of maturation of malignant cells is only feasible provided that the transformed cells have not lost their genetic competence for maturation. We therefore cannot rule out the possibility that in patient S.K., leukocyte development is arrested at a stage characterized by a unique ability to respond to the inducing properties of retinoic acid, possibly related to the hybrid (promyelocytic/T lymphocytic) phenotype.

NOTE ADDED IN PROOF

In relapse, the patient again presented as TdT-positive APL, but was t(15; 17) in all of the metaphases examined.

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