NB4, a Maturation Inducible Cell Line With t(15;17) Marker Isolated From a Human Acute Promyelocytic Leukemia (M3)

By M. Lanotte, V. Martin-Thouvenin, S. Najman, P. Balserini, F. Valensi, and R. Berger

Acute promyelocytic leukemia (APL) is a well-defined entity among acute leukemia, cytogenetically characterized by a t(15;17) (q22;q11-12) translocation. In vitro and in vivo studies suggest that all-trans retinoic acid (RA) treatment restores cell maturation. We have isolated the first permanent cell line with t(15;17), derived from the marrow of a patient with APL in relapse. The establishment of the cell line, its morphologic, karyotypic, and immunohistochemical features are reported. RA induced cell line maturation. Cells strongly expressed myeloid markers, but also some T-cell markers. Additional karyotypic abnormalities, a 12p rearrangement and the possible presence of a homogenous staining region (HSR) on 19q+ are discussed both in relation to T-cell (CD2, CD4) and monocyte (CD9) markers, and to the acquired cell growth autonomy. The cell line represents a remarkable tool for biomolecular studies.

IN MANY HEMATOPOIETIC malignancies specific chromosomal translocations correlate with the clinical, immunohistochemical, and morphologic features so that they are not only the hallmarks of the diseases but acknowledged promoting events in the pathogenesis. In particular the t(15;17) (q22;q11-12) associated with the acute promyelocytic leukemia (APL) of the French-American-British (FAB) classification, might be responsible for triggering the uncontrolled cell proliferation that correlates with cell maturation blockade.

That granulocyte colony-stimulating factor (G-CSF or CSF-3), myeloperoxidase, and retinoic acid (RA) receptor genes, and several proto-oncogenes might be located at, or in the vicinity of, the chromosomal breakpoint has urged investigators to evaluate the effects of t(15;17) in the expression of these genes and their role in APL cell proliferation and maturation. That APL respond to RA by a rapid growth arrest, as well as morphologic and functional maturation, suggests that the expression of some genes tightly implicated in RA signal transduction or in the maturation process might be altered by the translocation and justify the cloning of this region. Unfortunately, the absence of a genuine human promyelocytic cell line has impeded in vitro culture and biomolecular studies. Although HL60 has been called "promyelocytic" and, despite its response to RA, it is now admitted that it was derived from an acute myeloblastic leukemia with maturation (M3). Moreover, it does not contain the typical t(15;17).

In view of repeated failures to establish APL cell lines, we made efforts to carefully analyse APL cell growth patterns. Bone marrow cells from an APL patient in relapse were cultured for 14 weeks on a bone marrow stromal cell layer before the emergence of the autonomous cell line (NB4) occurred. We report here the features of this first ever isolated human cell line, with t(15;17) chromosomal marker. We also describe the pattern of differentiation/maturation of NB4 cells in response to all-trans RA treatment.

MATERIALS AND METHODS

Case history. N.B., a 20-year-old woman, was admitted in June 1986 to be treated for a typical APL (or M3 in the FAB nomenclature). At that time, her bone marrow was invaded by 72% abnormal hypergranular promyelocytes, and in the peripheral blood, the white blood cell count (WBC) was 2.4 x 10^9/L leukocytes without blast cells, 10 x 10^9/L platelets, 8.9 g/L hemoglobin. Disseminated intravascular coagulation (DIC) syndrome was marked. Complete remission was achieved by chemotherapy. From September 1987, the patient progressively relapsed and 70% of abnormal promyelocytes were observed in bone marrow in October. A second complete remission was achieved in December by chemotherapy, and the patient had a normal life under maintenance therapy. She relapsed for the second time in February 1989, with 62% abnormal promyelocytes in bone marrow, 1.9 x 10^9/L leukocytes in peripheral blood, and 27 x 10^9/L platelets. Cytogenetic studies, performed at that time from cultured (24 to 48 hours) bone marrow cells, showed complex changes with three types of karyotypes: 46, XX (1 metaphase), 46, XX, t(15;17)(q22;q11-12) (1 metaphase), and 79 to 88 chromosomes (16 metaphases) with a modal number of 87, some variation from cell to cell, and variable marker chromosomes. However, all the hypotetraploid cells exhibited the t(15;17) translocation, and a rear ranged No. 19. Representative karyotype could be written: 87, XXX, -X, -3, +5, -9, -10, -11, -14, -18, -19, -19, t(15;17)(q22;q11-12), t(15; 17), +der(19)(19;?)q(13;?), +4 mar. The patient was treated by all-trans RA, but no complete remission could be achieved. She died in May 1989. Five cytogenetic studies were performed with some variations from one to another, but always showing the presence of t(15;17) translocation and a variable proportion of cytogenetically normal metaphases. At the last examination, in May 1989, all 19 metaphases analyzed had the typical t(15;17) translocation and a rearranged No. 19.

Cell culture and establishment of the NB4 cell line. A sample of marrow was obtained from the patient with informed consent at the last karyotypic examination after the patient had received RA treatment. WBC were separated by centrifugation over Ficoll-Hypaque and cultured at 10^6 cell/mL on an allogeneic feeder layer of human bone marrow stromal cells, with RPMI 1640 medium.
PROMYELOCYTIC CELL LINE WITH \((t(15;17))\) MARKER

(GIBCO, Grand Island, NY) supplemented with 12.5% fetal calf serum (FCS), 7.5% donor serum (Flow Lab, Glasgow, Scotland), in plastic flask at 37°C in humidified air plus 5% CO₂. Compared with Gardner and Kaplan's method, \(^{20}\) hydrocortisone was omitted because we noticed a slight inhibitory effect on APL cells. Cultures were replenicshed each week by replacing one half of the growth medium with fresh medium. After 4 to 5 weeks the adherent layer containing foci of proliferating leukemic cells was detached with a teflon scraper and the whole cell population was transferred without dissociation in a flask containing a freshly established feeder of bone marrow stromal cells. Doing so, a progressive enrichment of proliferating APL cells was obtained. Microenvironment-dependent growth was observed during 14 weeks. Autonomous growth was then noticed and a rapidly expanding cell population overgrew the culture to become a cell line. APL cells cultured thereafter were designated NB4. Growth conditions of NB4 cells were improved by culturing cells at \(2 \times 10^8\) cell/mL in RPMI 1640 medium supplemented with 10% FCS alone (doubling time, 36 to 40 hours). So far, the NB4 cell line has been grown for more than 9 months; aliquots of cells were frozen at \(-80°C\).

Cytogenetic analysis. Chromosomes were studied when the cell line was established (August 1989) and after 40 weeks of culture with two passages a week. R bands with Giemsa after heating (RHG)-banding technique was applied and the chromosomes classified according to the international nomenclature.\(^{21}\)

Induction of differentiation. NB4 cells (\(10^7\)/mL), suspended in RPMI 1640 medium supplemented with 12.5% FCS alone, were incubated for 1 to 5 days in 96-well culture plates or in 24-well plates (Falcon, Oxnard, CA). Maturation was evaluated by microscopic examination of enzymic staining on histologic slides and also by the adhesion to either stromal cell extracellular matrix, fibronectin, or plastic culture surface. Cells were stained with May-Grunwald-Giemsa, myeloperoxidase, \(\alpha\)-naphthyl butyrate esterase, naphtol-ASD chloroacetate, alkaline phosphatase, and \(\beta\)-galactosidase, or plastic culture surface. Cells were stained with TruBlot (Coultronics, Marseille-Luminy, France); anti-TAC was kindly provided by Dr Ruan (University of Shanghai, Second Medical School).

RESULTS

Establishment, morphology, and cytometry of the cell line. APL cells were cultured on a narrow stromal cell layer. From the beginning of the culture, it became evident that a subpopulation of APL cells that had achieved some "instructive cooperation" with the hematopoietic microenvironment was being selected. The bulk population, with loose interactions with stromal cells, was hypergranular promyelocytes in G0/G1 phase, while few APL cells tightly associated to stromal cells showed mitotic figures, were much less granular, and had the broad morphology of blast cells found in APL. The latter, despite their leukemic origin, were reminiscent of the stromal cell-associated cells in long-term marrow culture.\(^{30,32}\) The number of blast cells increased in culture. At the isolation of the NB4 cell line, the culture consisted only of proliferating blast cells. Growth conditions, morphologic, and histochemical features of the cell line are reported in Fig 1. After 9 months of culture (about 155 cell doublings), no salient changes were noticed.

Cytogenetic studies. Cytogenetic analyses were performed at the initial stage of culture, at the isolation of the NB4 cell line (Fig 2), and after 9 months of culture. At the initial stage of the culture, all 23 metaphases examined were normal with karyotypic variations from cell to cell. Their chromosome numbers were between 68 and 90, most of them in the hypotetraploid range, with random losses. However, chromosome 19 loss and replacement of chromosome 21, either alone or in various combinations, was observed in more than 50% of the metaphases. Chromosome composition of the patient's sample was abnormal with karyotypic variations from cell to cell. The karyotype was hypotetraploid with loss of at least one copy of chromosomes 8, 9, 10, 11, and 14. Composite karyotype may

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Specificity</th>
<th>Positive Cells (%)</th>
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<tbody>
<tr>
<td>W6-32</td>
<td>HLA Class I</td>
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</tr>
<tr>
<td>DR</td>
<td>HLA Class II</td>
<td>N</td>
</tr>
<tr>
<td>J5</td>
<td>CALLA (CD 10)</td>
<td>N</td>
</tr>
<tr>
<td>My7</td>
<td>Pan-myeloid (CD13)</td>
<td>88</td>
</tr>
<tr>
<td>My9</td>
<td>Pan-myeloid (CD33)</td>
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<tr>
<td>IoN1</td>
<td>Granulocyte (CD15)</td>
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<tr>
<td>IOB2</td>
<td>Monocyte (CD9)</td>
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<td>M1</td>
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</tr>
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<td>My4</td>
<td>Monocyte (CD14)</td>
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<td>Monocyte, platelets (CD36)</td>
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</tr>
<tr>
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<td>Immature T cells (CD7)</td>
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<tr>
<td>T3</td>
<td>T cells (CD33)</td>
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<td>T10</td>
<td>Activated T cells (CD38)</td>
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<td>T11</td>
<td>SRBC receptor T cell (CD2)</td>
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<td>Leu 3a</td>
<td>Helper T cells, HIV receptor (CD4)</td>
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<td>B4</td>
<td>Pan-B cell (CD19)</td>
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<tr>
<td>B6</td>
<td>Mature B cell (CD23)</td>
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<td>Lymphoid and myeloid precursor cells (CD34)</td>
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<td>Gp II/III, megakaryocyte/platelets (CD41)</td>
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</tr>
<tr>
<td>SZ21</td>
<td>Gp IX, platelets (CD42)</td>
<td>N</td>
</tr>
<tr>
<td>OL67</td>
<td>Glycophorin, erythrocytes</td>
<td>N</td>
</tr>
</tbody>
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Indirect immunofluorescence staining on cell suspensions was analyzed by flow cytofluorometry performed on an EPICS Profile (Coultronics).

Abbreviations: HIV, human immunodeficiency virus; Gp, glycoprotein; N, negative.

Indirect immunofluorescence staining on cell suspensions was analyzed by flow cytofluorometry performed on an EPICS Profile (Coultronics).
be summarized as follows: 80-87,XXX,-X,-3,-8,-9,
-10,-12,-12,-14,-14,-18,-19,-19,der(12)t(12;?)p12;
?);t(15;17)(q22;q11-12),t(15;17),der(19)t(19;?),+ variable
markers. Because of this variation, it is difficult to establish
minor differences between karyotypes from “fresh cells”
and from the cell line NB4. However, a new der(12) marker
was consistently present in the cell line while it was not
found in “fresh” cells. Chromosome 19 rearrangement,
resembling hsr(19)q13), present in some metaphases of
fresh cells, was apparently selected during the establish-
mant of the cell line because it was present, most often
duplicated, in all metaphases (Fig 2).
**Immunocytologic analysis.** Cell surface markers of NB4 were analyzed (Table 1) and followed during 9 months, but no salient changes were found. It showed that NB4 expressed markers of the granulocytic lineage, but strikingly also some T-cell markers, like CD2, CD4, and a monocytic one like CD9. The percentages of positive cells for myeloid markers (73% to 89%) and T-cell markers (73% for CD4) suggest that features of both lineages are simultaneously expressed. CD9 was expressed by 73% of the cells, while cells were clearly negative for other monocyte-related markers like CD14 or CD36. These data are reminiscent of a hybrid leukemia.²⁴ Yet, the expression of these antigens might be related to unexpected gene activations linked to the multiple karyotypic alterations found. For example, the possible relationships between the 12p rearrangement and the localization of the genes encoding CD4⁴⁶-²⁸ and CD9,⁷ both expressed in NB4 cells, deserve attention.

**RA-induced maturation.** NB4 cells were treated with all-trans RA (1 μmol/L) for 6 days. Growth arrest occurred after 48 hours; morphologic maturation was accompanied by modifications of surface antigens and functional markers (Table 2). A strong increase of the $\alpha$-integrin-related marker, CD11b, was noticed; CD11c, absent on blast cells was strongly expressed on 75% of the differentiated cells (Table 2); these changes were associated with a marked increase in cell adhesiveness (Fig 1) to the substratum and extracellular matrices.³¹ Superoxide and hydrogen peroxide production and NBT reduction were quantitatively analysed with both enzyme-linked immunosorbent assay (ELISA) based micromethods (Table 2) and histochemistry (Fig 1); a strong enhancement of "oxidative" or "respiratory" burst indicates a potent microbicidal capacity corroborating RA-induced morphologic maturation. Finally, the myeloperoxidase, which was highly expressed in blast cells, was not significantly decreased during RA-induced maturation.
concluded that: permanent cell line. From our previous attempts, we pattern that probably conditioned failures in establishing permanent cell line with t(15;17) established from the cycles. Plating efficiency was as low as 50 per 10⁵ APL cells.

...microenvironmentation potential in vitro. Mitotic cells are often scarcely possibly in Go?) lasting several weeks, during which cells become hypergranular and show an intense myeloperoxidase reaction. However, APL cells do survive in culture, while many leukemia cell types die; it suggests that they have no requirement for survival factors, unlike normal promyelocytes or factor-dependent cell lines. (2) None of the so far tested hematopoietic growth factors (including G-CSF) support in vitro growth over more than 3 to 4 cell cycles. Plating efficiency was as low as 50 per 10⁵ APL cells. (3) APL cells achieved cell-cell or cell-matrix interactions with bone marrow stromal cells. Few microenvironment-associated APL cells (1 to 10 per 10⁵ plated cells) proliferate. These conditions were, in many cases, sufficient to maintain cultures in a steady state (cell renewal counteraing death) for several months. If the above observations are indeed valid, one would expect that marrow stromal cells/APL cocultured during several months would allow selection of growing cells. We reasoned that the isolation of a permanent APL/M₃ cell line depends on a second event responsible for growth autonomy and that its probability should considerably increase in the “fresh” material. Alternatively, a self-renewing leukemia progenitor cell population, already present as a minute component in the marrow of the patient could have indeed been selected in vitro. Several observations argue in favor of the latter hypothesis: (1) Among the multiple chromosome changes in the blast cells of the patient, perhaps partly related to previous treatments, some rearrangements favoring in vitro growth could have been selected. In this respect, the presence of a possible HSR on the 19q+ marker should be investigated further. (2) NB4 cells have retained morphologic features of a minor blast cell population found in the patient at relapse.

NB4 cells have a myeloblastic morphology without the typical APL granules. This finding does not necessarily contradict the promyelocytic feature of the leukemia, because hypogranular forms of APL had been described. It is very unlikely that a rapidly growing promyelocytic cell, if it does exist as such, could maintain a heavy load of granules (hypergranular) over numerous cell cycles. Our assumption is that the typical hypergranular promyelocytic cells found in M3 leukemia comprise an end-cell population derived from a more immature cell. The high frequency of relapse with myeloblastic morphology in patients with APL is consistent with the presence of such an immature cell population.

RA induced a rapid morphologic and functional maturation of NB4 cells; cell proliferation was no longer detectable after 3 days of a continuous treatment with RA. NB4 maturation with others inducers, like phorbol esters, dimethyl sulfoxide, cyclic nucleotides, and corticosteroids, never equalled in intensity and rapidity the RA-induced maturation (not shown). Each of these inducers, of course, merit specific investigations; possible synergistic effects

### Table 2. Maturation-Related Phenotypic Changes of NB4 Cells Induced by All-Trans RA

<table>
<thead>
<tr>
<th>Phenotypic Changes</th>
<th>Myeloblasts</th>
<th>Myelocytes</th>
<th>Metamyelocytes and polynuclear neutrophils</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>48 h</td>
<td>110 h</td>
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#### DISCUSSION

The cell line NB4 is, as far as we know, the only permanent cell line with t(15;17) established from the leukemic cells of a patient with APL.

Human APL (M3) cells achieve singular in vitro growth pattern that probably conditioned failures in establishing permanent cell line. From our previous attempts, we concluded that: (1) APL cells have a strikingly low proliferation potential in vitro. Mitotic cells are often scarcely detected after 3 to 4 days in culture. Cell cycle analysis by flow cytometry demonstrates a growth arrest in G₁ (or possibly in G₂?) lasting several weeks, during which cells become hypergranular and show an intense myeloperoxidase reaction. However, APL cells do survive in culture, while many leukemia cell types die; it suggests that they have no requirement for survival factors, unlike normal promyelocytes or factor-dependent cell lines. (2) None of the so far tested hematopoietic growth factors (including G-CSF) support in vitro growth over more than 3 to 4 cell cycles. Plating efficiency was as low as 50 per 10⁵ APL cells. (3) APL cells achieved cell-cell or cell-matrix interactions with bone marrow stromal cells. Few microenvironment-associated APL cells (1 to 10 per 10⁵ plated cells) proliferate. These conditions were, in many cases, sufficient to maintain cultures in a steady state (cell renewal counteraing death) for several months.

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between these inducers and cytostatic agents also justify detailed studies (manuscript in preparation).

In conclusion, the chief interest of this cell line lies in the tool it offers to molecular studies of t(15;17). Another major interest is, of course, its capacity to mature into functional polynuclear cells following RA treatment; it should help define the role of genes controlled (activated or suppressed) by RA signals in hematopoietic proliferation and differentiation. The cell line should also permit to explore the effects of various maturation inducers, growth factors, chemotherapeutic drugs, or even retroviral agents. A number of these investigations are currently in progress.

REFERENCES


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