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. Balerini, 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.

© 1991 by The American Society of Hematology.

From INSERM U-301, SDI No 159541 CNRS, Centre Hayem and IGM; and Laboratoire Central d’Hémato logie, Hôpital Saint-Louis, Paris, France.

Submitted August 1, 1990; accepted October 30, 1990.

Supported by INSERM, CNRS, the Fondation pour la Leucémie, and the Ligue Nationale contre le Cancer.

Address reprint requests to M. Lanotte, INSERM U-301, Centre G. Hayem, Hôpital Saint Louis, 27 rue Juliette Dodu, 75010-Paris, France.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1754 solely to indicate this fact.

© 1991 by The American Society of Hematology.

© 1991 by The American Society of Hematology.
PROMYELOCYTIC CELL LINE WITH t(15;17) MARKER

(JIBCO, Grand Island, NY) supplemented with 12.5% fetal calf serum (FCS), 7.5% donor horse serum (Flow Lab, Glasgow, Scotland), in plastic flask at 37°C in humidified air plus 5% CO₂. Compared with Gardner and Kaplan's method,26 hydrocortisone was omitted because we noticed a slight inhibitory effect on APL cells. Cultures were replenished 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 × 10⁵ 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 during 9 months; aliquots of cells were frozen at −80°C.

Cyto genetic 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 (10⁵/mL), suspended in RPMI containing 10% FCS and various concentrations of all-trans RA (Sigma-Chimie, La Verpillière, France) as an inducing agent, were incubated for 1 to 5 days in 96-microwell 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-Grünwald-Giemsa, myeloperoxidase, α-naphthyl butyrate esterase, naphth-ASD chloroacetate, alkaline phosphatase, and nitro blue tetrazolium reduction assay (NBT).

Cell surface markers. Indirect immunofluorescence staining on cell suspensions was performed as previously described. The flow cytometry analysis was performed on an EPICS Profile (Coultronics, France). A large panel of monoclonal antibodies (Table 1) has been used: B4, B6, J5, My7, My9 (Coultronics); DR, PanB (Dukopatts, Glostrup, Denmark); leu 3a, Leu 9, (Becton Dickinson, Mountain View, CA); I0B2, ION1 (Immunotech, Marseille-Luminy, France); anti-TAC was kindly provided by Dr Ruan (University of Shangai, Second Medical School).

RESULTS

Establishment, morphology, and cytomtery 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.2729 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 analyses. Cytogenetic analyses were performed at the initial stage of culture, at the isolation of the NB4 cell line (Fig 2). After 9 months of culture, all 23 metaphases examined were abnormal 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. All metaphases had t(15;17) (q22;q11-12) translocation associated with other rearrangements variable from one cell to another. However, chromosome 19 loss and replacement by 19q+ with extramaterial resembling homogenous staining region (HSR), on the one hand, and a der(12)t(12;?) (p12;?) were present in all the metaphases examined.

The t(15;17) translocation, present in the first karyotype performed on APL from the patient following the second relapse, was observed in all metaphases of the established cell line. Karyotypic complexity was observed both in the initial stage of culture, all 23 metaphases examined were abnormal with karyotypic variations from cell to cell. 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 (10⁵/mL), suspended in RPMI containing 10% FCS and various concentrations of all-trans RA (Sigma-Chimie, La Verpillière, France) as an inducing agent, were incubated for 1 to 5 days in 96-microwell 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-Grünwald-Giemsa, myeloperoxidase, α-naphthyl butyrate esterase, naphth-ASD chloroacetate, alkaline phosphatase, and nitro blue tetrazolium reduction assay (NBT).

Cell surface markers. Indirect immunofluorescence staining on cell suspensions was performed as previously described. The flow cytometry analysis was performed on an EPICS Profile (Coultronics, France). A large panel of monoclonal antibodies (Table 1) has been used: B4, B6, J5, My7, My9 (Coultronics); DR, PanB (Dukopatts, Glostrup, Denmark); leu 3a, Leu 9, (Becton Dickinson, Mountain View, CA); I0B2, ION1 (Immunotech, Marseille-Luminy, France); anti-TAC was kindly provided by Dr Ruan (University of Shangai, Second Medical School).

RESULTS

Establishment, morphology, and cytomtery 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.2729 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 analyses. Cytogenetic analyses were performed at the initial stage of culture, at the isolation of the NB4 cell line (Fig 2). After 9 months of culture, all 23 metaphases examined were abnormal 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. All metaphases had t(15;17) (q22;q11-12) translocation associated with other rearrangements variable from one cell to another. However, chromosome 19 loss and replacement by 19q+ with extramaterial resembling homogenous staining region (HSR), on the one hand, and a der(12)t(12;?) (p12;?) were present in all the metaphases examined.

The t(15;17) translocation, present in the first karyotype performed on APL from the patient following the second relapse, was observed in all metaphases of the established cell line. Karyotypic complexity was observed both in the initial stage of culture, all 23 metaphases examined were abnormal 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. All metaphases had t(15;17) (q22;q11-12) translocation associated with other rearrangements variable from one cell to another. However, chromosome 19 loss and replacement by 19q+ with extramaterial resembling homogenous staining region (HSR), on the one hand, and a der(12)t(12;?) (p12;?) were present in all the metaphases examined.

The t(15;17) translocation, present in the first karyotype performed on APL from the patient following the second relapse, was observed in all metaphases of the established cell line. Karyotypic complexity was observed both in the short term bone marrow culture and in the cell line with a wide variation from one cell to another. The karyotype was hypotetraploid with loss of at least one copy of chromosomes 8, 9, 10, 11, and 14. Composite karyotype may

---

Table 1. Cell Surface Antigens on NB4 Cells

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Specificity</th>
<th>Positive Cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>W6-32</td>
<td>HLA Class I</td>
<td>89</td>
</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>
<td>85</td>
</tr>
<tr>
<td>IoN1</td>
<td>Granulocyte (CD15)</td>
<td>89</td>
</tr>
<tr>
<td>IOB2</td>
<td>Monocyte (CD9)</td>
<td>73</td>
</tr>
<tr>
<td>M1</td>
<td>α-integrin, monocyte, granulocyte (CD11b)</td>
<td>34</td>
</tr>
<tr>
<td>Leu M5</td>
<td>α-integrin, monocyte, granulocyte (CD11c)</td>
<td>N</td>
</tr>
<tr>
<td>Leu M3</td>
<td>Monocyte (CD14)</td>
<td>N</td>
</tr>
<tr>
<td>My4</td>
<td>Monocyte (CD14)</td>
<td>N</td>
</tr>
<tr>
<td>M5</td>
<td>Monocyte, platelets (CD36)</td>
<td>N</td>
</tr>
<tr>
<td>Leu 9</td>
<td>Immature T cells (CD7)</td>
<td>N</td>
</tr>
<tr>
<td>T3</td>
<td>T cells (CD3)</td>
<td>N</td>
</tr>
<tr>
<td>T10</td>
<td>Activated T cells (CD38)</td>
<td>24</td>
</tr>
<tr>
<td>T11</td>
<td>SRBC receptor T cell (CD2)</td>
<td>65</td>
</tr>
<tr>
<td>Leu 3a</td>
<td>Helper T cells, HIV receptor (CD4)</td>
<td>73</td>
</tr>
<tr>
<td>TAC</td>
<td>β-Chain IL-2 receptor</td>
<td>N</td>
</tr>
<tr>
<td>B4</td>
<td>Pan-B cell (CD19)</td>
<td>N</td>
</tr>
<tr>
<td>B6</td>
<td>Mature B cell (CD23)</td>
<td>N</td>
</tr>
<tr>
<td>HPCA1</td>
<td>Lymphoid and myeloid precursor cells (CD34)</td>
<td>N</td>
</tr>
<tr>
<td>SZ2</td>
<td>Gp II/III, megakaryocyte/platelets (CD41)</td>
<td>N</td>
</tr>
<tr>
<td>SZ21</td>
<td>Gp IX, platelets (CD42)</td>
<td>N</td>
</tr>
<tr>
<td>OI67</td>
<td>Glycoporphin, erythrocytes</td>
<td>N</td>
</tr>
</tbody>
</table>
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 establishment of the cell line because it was present, most often duplicated, in all metaphases (Fig 2).
PROMYEOCYTIC CELL LINE WITH t(15;17) MARKER

Fig 2. Example of karyotype of NB4 cell line (R-bands). Arrows indicate the rearranged chromosomes.

**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. 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 CD4 and CD9, 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 α-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. Superoxide and hydrogen peroxide production and NBT reduction were quantitatively analyzed 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.
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 G0 (or possibly in G0?) 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 10⁶ 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 10⁶ plated cells) proliferate. These conditions were, in many cases, sufficient to maintain cultures in a steady state (cell renewal counterbalancing 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 a cycling population compared with a G₀ arrested one. This strategy was adopted to isolated the NB4 cell line. The primary culture of leukemic cells on a stromal cell layer furnished a conducive environment for cell growth. We cannot conclude whether an additional mutation was responsible for the generation of the autonomous leukemic cell clone that developed in the culture of microenvironment-dependent APL cells. It has to be noted that an additional der(12) marker was present in the cell line but not detected 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...
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 polyvuclear 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.

ACKNOWLEDGMENT

The authors thank C. Chomienne, M.T. Daniel, I. Dobo, M. Le Coniat, R. Miglierina, S. Ruchaud, and Pr. F. Teillet for their help during the achievement of this work. Requests for the NB4 cell line should be addressed to M.L.

REFERENCES


NB4, a maturation inducible cell line with t(15;17) marker isolated from a human acute promyelocytic leukemia (M3)

M Lanotte, V Martin-Thouvenin, S Najman, P Balerini, F Valensi and R Berger