Comparative Cytogenetic Studies of Normal and Leukemic Lymphoblastoid Cell Lines during the Course of Their Establishment

A. M. Vénuat, C. Rosenfeld, and M. J. Testu

ABSTRACT

A comparative chromosomal analysis was made of 10 human lymphoblastoid cell lines, four of which originated from normal donor lymphocytes and six of which were from leukemic peripheral blood. For comparison of lymphoblastoid cells with respect to their normal or leukemic origin, cytogenetic studies have been carried out regularly since the beginning of the culture during more than 3 years. Samples were drawn during the three phases previously described for the establishment of these lines. The chromosome distribution remained diploid for at least 2 years in normal cell lines, and the cells were euploid. In contrast, an important variability of the chromosome set was demonstrated during the same period in leukemic cell lines. Moreover, in these lines, it was always possible to observe a nonsystemic pseudodiploidy. After 2 years, a clonal evolution was described in both types of cell lines that carried at least one marker. With a controlled-heating denaturation technique, it was possible to identify the markers as specific to each cell line. The cells with marker chromosomes appeared to have a selective advantage of growth.

INTRODUCTION

Spontaneous in vitro establishment of leukocyte lines that originate from normal and leukemic human blood is now shown to occur quite frequently. Many cytogenetic studies of such lymphoblastoid cell lines have been reported (8-10, 16). The results are contradictory but, taken together, do not seem to show any significant differences between normal and leukemic lines.

The majority of these studies were unable to show whether abnormalities found in the cultures were already present in the original cell population or whether they were induced in vitro, as has been suggested for other types of cell cultures (1, 6).

The role of herpes-type viruses in the appearance of chromosomal abnormalities in such cell lines has been discussed (5, 7, 24). Recently, Steel et al. (18) described the late cytogenetic evolution of a clonal type.

Therefore, we have studied (22, 23) the in vitro cytogenetic evolution of 10 permanent human normal and leukemic lymphoblastoid lines by investigating them at regular intervals over a period of approximately 4 years starting from the time of seeding .

Moreover, we have used the R-band technique that allows us to determine the abnormalities and sometimes the mechanism of their formation. Preliminary results have already been reported (20, 21).

MATERIALS AND METHODS

Cultures

The cultures were seeded without phytohemagglutinin from the human blood lymphocytes of normal donors (LHN1, LHN13, LHN16, LHN30); or from leukocytes of patients with acute myeloid leukemia in relapse (HER, DUK, HUE, BAR, GOZ), or with chronic myeloid leukemia in blastic crisis (KRY). The leukemic blood cell samples were taken aseptically with a heparinized syringe and then sedimented at 37° for 1 hr. After the plasma had been centrifuged for 5 min at 800 rpm, the cells were washed twice with 0.9% NaCl solution. The cell pellet was resuspended in complete medium, and a count was made before definitive seeding at the concentration of 2 to 3 x 10⁶ cells/ml. Samples of normal leukocytes were obtained after differential centrifugation on an IBM machine. This technique enables us to obtain WBC with very few erythrocytes and polymorphonuclear leukocytes. The 1 disadvantage is the presence of many platelets, which necessitates washing the cells twice in 0.9% NaCl solution.

The cells were cultivated in Roswell Park Memorial Institute Medium 1640 supplemented with 20% fetal calf serum (Grand Island Biological Co., Grand Island, N.Y.); penicillin, 100 units/ml; and streptomycin, (50 µg/ml). Cultures were refed twice a week; counts were made under a hemocytometer with the trypan blue exclusion method. When cultures were established as normal cell lines, they were readjusted at each refeeding to a concentration of 5 x 10⁶ cells/ml. A typical curve representing the kinetics of establishment of a line is presented in Chart 1 with the total cell number (ordinate) as a function of time (abscissa). This cumulative curve of growth has been obtained by plotting the total cell number found in each flask on semilogarithmic paper. Once established, this curve was made by multiplying the ratio between the number of cells at the time of transfer and the respective inoculum by the preceding cell number on the curve. As can be seen from the curve, the kinetics of the establishment followed 3 periods. After the cells were seeded into culture (Tn), the first period corresponds to a phase (Tn) during which cell death predominates. The
second period is characterized by an irregular multiplication phase ($T_e$) which is followed by a third period in which the cell growth is exponential ($T_E$) (15).

Cytogenetic Study

Classical Technique. At $T_2$, the leukemic cells were cultivated for 24 and 48 hr without phytohemagglutinin, while normal cells were cultivated for 72 hr with phytohemagglutinin (Difco Laboratories, Detroit, Mich.). At $T_2$, $T_4$, and $T_E$, samples of 5 to 10 × 10⁶ cells were withdrawn from the culture flasks. The fixation and staining techniques of De Grouchy et al. (2) or Lejeune (19) were applied. Fifty to 100 mitoses or more were photographed per sample. After the chromosomes were counted, a histogram was drawn, and 20 to 25 metaphases were chosen to establish the karyotypes.

Controlled-Heating Denaturation Technique, R-band Technique (3). After fixation, the slides were placed in a phosphate-buffered solution (20 mm) at pH 6.5 and maintained at 86°C for 5 to 10 min. They were rinsed with cold water and stained with 4% Giemsa solution (Giemsa R; RAL, Paris, France).

The film used was Recordak AHU 5786 microfilm (Kodak, Paris, France) with a photomicroscope I (Carl Zeiss, Oberkochen/Württ, West Germany). The classification of chromosomes was made according to the Chicago (1966) or Paris (1971) conferences.

RESULTS

No mitoses were found at $T_D$ in cell lines of either normal or leukemic origin.

Normal Cell Lines

Histograms showing the distribution of chromosome numbers in cell lines LHN₄, LHN₁₃, LHN₁₆, and LHN₁₉ are given in Chart 2. The modal peak for the number of chromosomes estimated at different times is 46 for all the normal lines except LHN₁₆. The latter, which was still diploid after 18 months in culture, showed a mode of 47 chromosomes at 33 months. The histogram of this line is not represented at $T_2$, because the total number of mitoses was insufficient for inclusion, although the majority were diploid.

LHN₄ Line. The cells were euploid until 18 months. They had a normal karyotype (46,XY). By use of classical techniques it was found that at 33 months, when the cell population was at 47 chromosomes, the karyotypes showed a supernumerary element like a No. 3 chromosome. This result was confirmed with the denaturation technique (Fig. 1a). The remaining part of the karyotype did not show any modifications (47,XY,+3). At 42 months we observed a new evolution. The cells had at this time a chromosomal mode of 48. They still had the supernumerary No. 3 chromosome, but they had also acquired another element in the C group. The controlled-heating technique demonstrated that it was a No. 12 chromosome (48,XY,+3,+12) (Fig. 1b).

LHN₁₃ Line. From $T_2$ until 24 months, the cells were euploid with a normal karyotype (46,XY), confirmed upon denaturation (Fig. 2). At 31.5 months and thereafter (33, 34, and 37 months), it could be seen that chromosome markers appeared, Dq+, Mqh, and Cqh (Fig. 3) with variations in frequency (Table 1). From this Table it can be seen that, at 31.5 months, the Cqh element is the most frequent and is related to Dq+ and to Mqh. At 33 months, Dq+ is proportionally high and Cqh is associated with it, but not with Mqh, which is unusual at this time. In contrast, after 34 months of culture a very high frequency of Mqh alone or associated with Cqh and a decreasing rate of Dq+ were observed. At 37 months, most metaphases presented the 2 Mqh and Cqh markers. Furthermore, the association of Dq+, Mqh, and Cqh could be seen only at this time. Table 2 shows the percentage of mitoses with each marker either alone or associated. It is clear that the decrease in Dq+ is simultaneous with the increase in Mqh. On the other hand, the Cqh element had a relatively stable frequency and was
**Table 1**

<table>
<thead>
<tr>
<th>Time (mos.)</th>
<th>Dq+</th>
<th>Mqh</th>
<th>Cqh</th>
<th>Dq+,Mqh</th>
<th>Dq+,Cqh</th>
<th>Mqh,Cqh</th>
<th>Dq+,Mqh,Cqh</th>
</tr>
</thead>
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<tr>
<td>31.5</td>
<td>2.7</td>
<td>18.9</td>
<td>8.1</td>
<td>51.4</td>
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<td>13.5</td>
<td>5.4</td>
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<tr>
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<td>0</td>
<td>43.7</td>
<td>4.2</td>
<td>12.5</td>
<td>2.1</td>
<td>37.5</td>
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<td>34</td>
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<td>2.55</td>
<td>43.6</td>
<td>7.7</td>
<td>2.55</td>
<td>0</td>
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<tr>
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<td>11.11</td>
<td>0</td>
<td>14.82</td>
<td>18.52</td>
<td>0</td>
<td>1.85</td>
<td>50</td>
</tr>
</tbody>
</table>

**Table 2**

<table>
<thead>
<tr>
<th>Time (mos.)</th>
<th>Dq+</th>
<th>Mqh</th>
<th>Cqh</th>
</tr>
</thead>
<tbody>
<tr>
<td>31.5</td>
<td>32.4</td>
<td>13.5</td>
<td>70.3</td>
</tr>
<tr>
<td>33</td>
<td>83.3</td>
<td>6.3</td>
<td>50</td>
</tr>
<tr>
<td>34</td>
<td>5.1</td>
<td>89.7</td>
<td>51.3</td>
</tr>
<tr>
<td>37</td>
<td>5.5</td>
<td>68.5</td>
<td>62.1</td>
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</table>

related indiscriminately to Dq+ (31.5 and 33 months) or to Mqh (34 and 37 months). These fluctuations in marker frequency indicated the nonstability of this line, which continued its clonal evolution. Only now have denaturation assays enabled us to identify the Dq+ as a 14q+ and the Cqh as a 10q+.

**LHN	extsubscript{16} Line.** At $T_a$ and $T_f$ (4 months) and $T_g$ (6 months), the samples studied showed euploid cells (46,XY), a result confirmed by denaturation.

**LHN	extsubscript{19} Line.** At $T_a$ and $T_f$ (2 months) and at 4 months the cells had a normal karyotype (46,XY) as demonstrated by both classical and denaturation techniques.

Of these 4 cell lines of normal origin, 1 was 37 months old (LHN	extsubscript{16}) and 1 was 42 months old (LHN	extsubscript{19}). By comparing them it was possible to affirm that for a period of 18 to 24 months the cells maintained their original normal karyotype. After this time certain lines (LHN	extsubscript{16}, LHN	extsubscript{19}) followed an evolution in which normal (LHN	extsubscript{16}) or abnormal (LHN	extsubscript{19}) marker chromosomes appeared. Thus these 2 lines seemed to continue their evolution without having acquired a stable karyotype.

**Leukemic Cell Lines**

Chart 3 shows the distribution of chromosome numbers in 6 cell lines. Except for the GOZ line, the lines showed a very heteroploid distribution (KRY at 2 months, HUE at 43 months) followed by a return to a diploid state or paradiplloid and hypodiploid phases (BAR at $T_a$ and 9 months, HER at 3 and 4 months). They also showed a peak at 46 chromosomes and another tetra- or paratetraploid phase (BAR at 17 and 20 months, HUE at 44 and 47 months). Cells of the DUK line had only 45 chromosomes in most distributions at 41 months. The establishment of the karyotypes enabled us to observe the consistent presence of pseudodiploid cells in all the samples studied, including GOZ at $T_a$, $T_f$, and $T_g$. This pseudodiploidy is not, in any case, systematized (Table 3).

**HER Line.** At 3 months 30% of metaphases had a pseudodiploid karyotype, and at 4.5 months 37% of cells were aneuploid.

**HUE Line.** The HUE line is detailed elsewhere (20, 21). At establishment (2 months) and after 6 months in culture, the cells presented 25 and 20% aneuploidy, respectively, with no systematic gain or loss of normal elements.

At 27 months of culture examination revealed many endomitoses, and, in 100% of metaphases, the absence of 1 C and 1 F chromosome, as well as the presence of 2 markers called Cq+ and Fp- q−. At 35 months a despiralization of distal parts of the long and short arms of 1 No. 3 chromosome appeared in some cells and on the distal part.
of short arms of some B chromosomes. Thus, between the early and late examinations, the number of abnormal metaphases increased so that by 38 months 100% of the cells had the 2 markers and 3qh or ph and a Bph. On such samples, with controlled-heating denaturation it has been possible to identify the Cq+ as 6q +, and Fp− q− as a 20p− q−, a chromosome 3 as a 3qh and a chromosome B as a 4ph or p+. When the two No. 6 chromosomes were missing, they were replaced by two 6q+ chromosomes which often associate themselves to form a dicentric chromosome.

At 44 months another examination revealed the stability of this karyotype with the same structural rearrangements. Furthermore, a sample that had been frozen at 35 months showed the same evolution.

The study of the HUE line demonstrates a late evolution of the clonal type which finally results in 100% of the cells showing a well-defined and stable karyotype over many generations.

**DUK Line.** At 3, 4, and 8 months, pseudodiploid cells represented 19, 22, and 25% of the population, respectively. At 25 months this percentage increased to 66%, at which point a marker chromosome appeared, one 2q+ chromosome taking the place of a normal No. 2 chromosome. By 41 months the whole population was aneuploid. The modal value was 45 chromosomes with a systematic loss of 1 G or of the Y. Upon examination after denaturation at 47 months, the 2q + chromosome was present in 100% of cells, with a 100% loss of No. 18 and Y chromosomes, sometimes with a poorly defined supernumerary (Fig. 4).

**BAR Line.** At 44 months another examination revealed the stability of this karyotype with the same structural rearrangements. Furthermore, a sample that had been frozen at 35 months showed the same evolution.

The study of the HUE line demonstrates a late evolution of the clonal type which finally results in 100% of the cells showing a well-defined and stable karyotype over many generations.

**KRY Line.** This line was originated from chronic myeloid leukemia blood cells in blastic crisis. At 70, 26.7% of cells put in culture were pseudodiploid. At 70 no mitoses were observed and there was no cellular division (14). Cell multiplication and appearance of DNA synthesis coincided with the beginning of Tt. However, in the leukemic lines originated either from acute myeloid leukemia or from blast crisis in chronic myeloid leukemia, a mixture of euploid and aneuploid cells was present at Tt, which was again found at the start of the first multiplication (Tt). This fact should be emphasized, taking into account that all leukemic blood used contained nonleukemic cells capable of competitive growth with the leukemic ones. Moreover, both types of culture were set without phytohemagglutinin in the same medium that is now known to enable pure leukemic cells to grow (13). The general fact observed in the 6 leukemia-originated cell lines is that there is a certain percentage of aneuploid cells, which is independent of the initial differing proportion of normal blood cells. The percentage of euploid cells, the competitive growth, the chemotherapeutic treatment of patients, and the timing of cell harvest are all differing factors to be taken into consideration. Whatever the cause of chromosomal abnormalities, there is a high incidence (18 to 50%) in these leukemic lines. This mixture of euploid and aneuploid cells can be found up to 2 years after initial seeding. This suggests that a leukemic donor, as opposed to a normal donor, has abnormal cells capable of division at all phases of culture and that the abnormalities do not merely develop or arise in vitro after establishment, as has been suggested by Sandberg et al. (17).

Moreover, the initial (T0) and final (Tf) mixtures of normal and abnormal karyotypes do not fit, at least in our cultures,
REFERENCES


Fig. 1. Karyotype of LHNr cells with R-band technique, a. at 33 months (47,XY,+3); b. at 42 months (48,XY,+3,+12).
Fig. 2. Karyotype of LHNr cells at 24 months after R-band technique (46,XY).
Fig. 3. LHNr mitoses at 31.5 months showing the 3 markers Dq+ and Cqh (a) and Mqh (b) with classical technique.
Fig. 4. Karyotype of DUK cells at 47 months after heating denaturation showing 2q+, losses of 1 No. 18 chromosome and the Y chromosome with a nonidentified marker.
Fig. 5. Karyotype of GOZ cells at 21 months, 20 days with R-band technique showing the supernumerary No. 12 chromosome with no systematic loss or acquisition in other group (here loss of 1 Chromosome 13 and acquisition of a third No. 20 chromosome can be seen).
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