Coexistence of two distinct cell populations (CD56+TcRγδ+ and CD56−TcRγδ−) in a case of aggressive CD56+ lymphoma/leukemia

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ABSTRACT

Background and Objectives. Large granular lymphocytes derive from two major lineages: one expressing the CD3 surface antigen (T-lymphocytes), and the other lacking this marker (NK-cells). Although developmental overlaps between natural killer cells and T-cells have been described, malignancies derived from these two cell types are considered as distinct lymphoid disorders.

Design and Methods. We report the case of a 30-year old man affected by a lymphoma/leukemia syndrome presenting with hepatosplenic lymphoma which rapidly transformed into aggressive NK-leukemia. Extensive flow cytometry studies and molecular analysis were repeated during the course of the disease, and showed an unexpected changing pattern.

Results. At diagnosis, flow cytometry analysis showed the co-existence of two cell populations, one CD56+, CD3+, TcRγδ+/H9253/H9254+, and the other CD56+, CD3− and TcRγδ−. Molecular analysis showed that the TcR genes had the same clonally rearranged pattern involving β, γ and δ genes in both populations. At disease relapse and during the terminal refractory phase, only CD3− cells were present.

Interpretation and Conclusions. This is an unusual case of CD56+ aggressive lymphoma/leukemia characterized by the clonal expansion of two phenotypically different cell populations, variably balanced during the course of the disease. The presence of the same TcR genomic rearrangement suggests the origin from a common progenitor able to differentiate along both T- and NK-pathways.©2000, Ferrata Storti Foundation

Key words: LGL leukemia, TcRγδ-NHL, NK leukemia, flow-cytometry.

N atural killer (NK) cells are defined by a characteristic immunophenotype and specific functional aspects. In the past years they have frequently been identified as large granular lymphocytes (LGL). It is now clear that LGLs belong to two major lineages, one expressing the CD3 surface antigen and the other lacking this marker, and that CD3+ and CD3− LGLs may share a complex surface phenotype including CD8, CD2, CD16, CD56 and CD57.

Malignant clonal proliferations have been described for both CD3+ and CD3− non-B-lymphocytes with their responsive clinical courses being chronic/indolent or acute/aggressive. In this paper we report on a patient affected by a CD56+ proliferative disorder characterized by the presence of two distinct cell populations, one CD3+/TcRγδ+/CD56+ and the other CD3−/TcRγδ−/CD56−. Cell morphology was insufficient to permit a confident classification of this case, while flow cytometry performed on peripheral blood and bone marrow cells played a pivotal role, as reported for other cases. The clinical picture was a mixture of hepatosplenic TcRγδ− T-cell lymphoma and aggressive NK-leukemia.

The aims of this study were: i) to characterize the two cell components; ii) to verify their clonal origin; iii) to evaluate, in different phases of the disease, the existence of a correlation between the clinical picture and predominance of one or the other cell population.

Case report

A 30-year old Caucasian male was admitted to hospital in February 1995 because of fever, fatigue, anemia and lymphocytosis. Physical examination showed jaundice, liver and spleen enlargement (6 and 14 cm below the costal margin, respectively), with no palpable lymph nodes. Blood counts showed hemoglobin 7.8 g/dL, white blood cells 15.4×10⁹/L, platelets 143×10⁹/L and reticulocytes 207×10⁹/L. A blood smear revealed 58% atypical lymphoid cells (Figure 1) and several circulating erythroblasts. Myeloperoxidase staining of circulating atypical cells was negative. A summary of the laboratory investigations is presented in Table 1; elevated serum lactate dehydrogenase, polyclonal hypergammaglobulinemia and moderate hyperbilirubinemia were the main
Bone marrow aspirate showed erythroid hyperplasia and minimal (7%) atypical lymphoid cell infiltration; hemophagocytic images were present. Although morphology (immature small agranular lymphocytes) by itself did not suggest an LGL proliferation, the immunophenotype was typical for such a disorder (Table 2). Ploidy analysis, performed on circulating leukemic cells by FACS with propidium iodide stain, showed a normal DNA content with a high fraction of "S" phase cells. Immunohematologic and serologic studies, including Coombs’ test and antibodies to HTLV I/II and EBV, were all negative. A CT scan showed intra-splenic focal areas. A fine needle aspirate from the spleen suggested large cell non-Hodgkin’s lymphoma.

In view of the patient’s very poor clinical condition, treatment with prednisone (1 mg/kg) was started immediately. The initial response was excellent, with improvement of the clinical status, spleen size reduction and a rise in hemoglobin; however, the disease progressed within two weeks. At this time, a repeat cytofluorimetric study showed a reduction of the CD3+ cell population (Table 2), associated with high expression of the Gp170 molecule (an antigen not tested previously, detected by the MRK16 MoAb).

Chemotherapy using the MACOP-B regimen was started, but was discontinued 4 weeks later because of signs of disease progression. Second line combination chemotherapy included ifosfamide, cytarabine, high-dose methotrexate and methylprednisolone. The treatment was highly effective and tolerance was excellent. Nevertheless, three weeks later a bone marrow aspirate showed complete marrow replacement by lymphoid blast cells. The patient started third line chemotherapy with of fludarabine, cytarabine, idarubicin and etoposide, combined with continuous infusion of cyclosporin-A as Gp170 revertant. On day +14 filgrastim was added. On day +21 there was marked clinical and hematologic improvement; bone marrow aspirate showed complete remission which was confirmed by FACS and molecular analysis.

Allogeneic bone marrow transplant from an HLA identical sister was planned, but leukemic relapse occurred after a few weeks and the patient was readmitted with high grade fever and liver and spleen enlargement. A repeat course of polychemotherapy identical to the previous one proved to be totally ineffective, and the patient died in August because of disease progression.

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**Table 1. CD56+ lymphoma/leukemia. Summary of laboratory investigation.**

<table>
<thead>
<tr>
<th>March</th>
<th>May</th>
<th>June</th>
<th>July</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb (g/dL)</td>
<td>7.8</td>
<td>8.3</td>
<td>12.4</td>
</tr>
<tr>
<td>WBC (&lt;10^9/L)</td>
<td>15.4</td>
<td>2.0</td>
<td>11.1</td>
</tr>
<tr>
<td>Plt (&lt;10^9/L)</td>
<td>143</td>
<td>114</td>
<td>114</td>
</tr>
<tr>
<td>Reticulocytes (&gt;10^9/L)</td>
<td>325</td>
<td>250</td>
<td>140</td>
</tr>
<tr>
<td>Total bilirubin (mg/dL)</td>
<td>2.44</td>
<td>0.95</td>
<td>0.83</td>
</tr>
<tr>
<td>ESR (mm/h)</td>
<td>120</td>
<td>100</td>
<td>33</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>99</td>
<td>43</td>
<td>129</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>43</td>
<td>134</td>
<td>60</td>
</tr>
<tr>
<td>γGT (U/L)</td>
<td>93</td>
<td>106</td>
<td>69</td>
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<tr>
<td>LDH (U/L)</td>
<td>4,428</td>
<td>4,224</td>
<td>473</td>
</tr>
<tr>
<td>IgG (mg/dL)</td>
<td>1810</td>
<td>871</td>
<td>524</td>
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<tr>
<td>IgA (mg/dL)</td>
<td>387</td>
<td>194</td>
<td>33</td>
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<tr>
<td>IgM (mg/dL)</td>
<td>156</td>
<td>48</td>
<td>14</td>
</tr>
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**Table 2. Immunophenotypic characterization of patient's bone marrow cells.**

<table>
<thead>
<tr>
<th>March</th>
<th>May</th>
<th>June</th>
<th>July</th>
</tr>
</thead>
<tbody>
<tr>
<td>% cells in lymphocyte gate</td>
<td>21</td>
<td>88</td>
<td>9</td>
</tr>
<tr>
<td>CD3+</td>
<td>48</td>
<td>4</td>
<td>85</td>
</tr>
<tr>
<td>Tcrγδ+</td>
<td>26</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Tcrαβ+</td>
<td>23</td>
<td>2</td>
<td>85</td>
</tr>
<tr>
<td>CD2+</td>
<td>70</td>
<td>98</td>
<td>90</td>
</tr>
<tr>
<td>CD7+</td>
<td>20</td>
<td>44</td>
<td>NT</td>
</tr>
<tr>
<td>CD5+</td>
<td>27</td>
<td>1</td>
<td>80</td>
</tr>
<tr>
<td>CD4+</td>
<td>14</td>
<td>9</td>
<td>4</td>
</tr>
<tr>
<td>CD8+</td>
<td>50</td>
<td>77</td>
<td>80</td>
</tr>
<tr>
<td>CD16+</td>
<td>55</td>
<td>22</td>
<td>3</td>
</tr>
<tr>
<td>CD56+</td>
<td>50</td>
<td>97</td>
<td>17</td>
</tr>
<tr>
<td>CD57+</td>
<td>4</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>CD56* Tcrγδ+</td>
<td>26</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>CD16* Tcrγδ+</td>
<td>26</td>
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<td>0</td>
</tr>
<tr>
<td>CD8* Tcrγδ+</td>
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</tr>
<tr>
<td>CD34+</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>CD13+</td>
<td>3</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
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<td>18</td>
</tr>
<tr>
<td>CD14+</td>
<td>2</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>CD19+</td>
<td>2</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

Values are in % of positive cells in the gate. In bold: most relevant data. NT: not tested.
Design and Methods

Immunophenotypic analysis

Cell surface antigens were tested by flow cytometry (Cytorun Absolute, Ortho Diagnostic Systems, USA). The membrane expression of differentiation antigens was investigated with a panel of monoclonal antibodies (MoAb) directly coupled with fluorescein (FITC) or phycoerythrin (PE), including OKT3-FITC (CD3), OKT11-FITC (CD2), Leu9-FITC (CD7), Leu1-FITC (CD5), OKT8-FITC (CD8), OKT4-FITC (CD4), Leu19-PE (CD56), Leu7-FITC (CD57), OKNk-FITC (CD16), TcRg6-FITC, TcRy6-FITC, HPCA2-PE (CD34), OKM13-FITC (CD13), LeuM9-PE (CD33), LeuM3-FITC (CD14), HD37-PE (CD19). The MoAb antibody was used for gp170 detection. Antibodies of the OK series were obtained from Ortho Diagnostic Systems, Raritan, NJ, USA. Leu panel, anti-CD34 and anti-TcR MoAbs were purchased from Becton Dickinson, San Jose, CA, USA. 

DNA studies

Mononuclear cells from bone marrow samples withdrawn at diagnosis and during the follow-up and from peripheral blood during disease progression were analyzed for β, γ, and TcR gene rearrangement by Southern blotting. High molecular weight genomic DNA was extracted using the standard phenol/chloroform method. Aliquots of 15 µg of genomic DNA were digested with 50 U of the appropriate restriction enzymes (all purchased from New England Biolabs, Beverly, MA, USA), electrophoresed on 0.8% agarose gel, and blotted on positively charged nylon membranes (Hybond N-plus, Amersham, UK). Following transfer, DNA was immobilized onto the membrane by incubation at 60°C in a vacuum oven, and alternatively hybridized to 50 ng of one of three radioactively labeled genomic probes. To study rearrangement of the gene encoding the β chain of TcR, we used the Cβ probe, a 1.1-Kb Eco RI-Hind III DNA fragment which recognizes both Cβ1 and Cβ2 sequences. Rearrangements of the genes encoding the γ and δ chain of the TcR were studied with Jγ 1.3 and Jδ 1 probes, respectively. The Jγ 1.3 probe, a 0.8-Kb Eco RI-Hind III genomic DNA fragment which recognizes both Jγ 1.3 and Jγ 1.2 gene segments, detects all rearrangements of TcRγ gene in Kpl restriction enzyme digests. The Jδ 1, a 0.5-Kb SacI-Eco RI genomic DNA fragment, which recognizes a Jδ 1 gene segment, detects TcRδ gene rearrangements and deletion.

Cell line production

We succeeded in establishing two different cell lines that we called DERL-NK2 and DERL-NK7. The immunophenotype was CD56+/CD3+/TcRγδ+ for DERL-NK2, from this patient, obtained from circulating leukemic cells, and CD56+/CD3+/TcRγδ- for DERL-NK7, obtained from bone marrow during the progression phase. They were thus reminiscent of the two main cellular components of the disease. Detailed data concerning these cell lines are the subject of a manuscript in preparation.

Results

Immunophenotypic analyses

The results of the immunophenotypic characterization performed on bone marrow nucleated cells (BM NC) on four different occasions are summarized in Table 2. The first study was performed at diagnosis. The cytometric gate was imposed on lymphocytes (lymphocyte gate), and included 21% of BM NC. A significant increase of CD56+ cells (50% in the gate) was found; within this fraction, two different cell populations could be identified, the first characterized by CD3, TcRγδ, CD56, CD16, CD8 and CD2, and the second expressing CD56, CD16, CD8 and CD2 in the absence of CD3 and TcR γδ or β. The flow cytometry pattern of TcRγδ (Figure 2, panel A), as well as dual fluorescence experiments, demonstrated that these two cell populations belonged to two distinct subsets. These findings were confirmed on peripheral blood cells (data not shown). The second typing was performed during disease progression under steroid treatment. At this time the gate included 88% of BM NC and was composed of 97% CD56+ cells, and the CD56+/TcRγδ+ population was confined to 3% of the events analyzed (Figure 2, panel B). Even in this phase, CD56+TcRγδ+ and CD56–TcRγδ– cells clearly belonged to two distinct cell populations. In June the patient achieved a clinical remission that was characterized by the presence, in the lymphoid gate, of 85% normal TcRγδ+ lymphocytes, and by the absence of TcRγδ– cells. At the final relapse, in July, BM NC were retested, and a dramatic predominance of CD56+TcRγδ+ cells was again detected in the lymphocyte gate.

Cytogenetics

Karyotype analysis was performed on bone marrow cells at diagnosis and during disease progression. At diagnosis, out of 28 metaphases analyzed, 16 were normal (46XY), and 12 were abnormal: 47XY+i(7q) [10]/48 XY+i(7q) [8]. Considering that at the time of this investigation the bone marrow contained less than 10% atypical lymphoid cells, the detection of 42% mitoses with a clonal marker indicates a high proliferative rate of the neoplastic population. This finding was confirmed by cell-cycle cyometric analysis, which showed 17% cells in S-phase. In May a repeat cytogenetic analysis showed six metaphases with a karyotype 47 XY +i(7q) and three with 46XY.

Molecular analysis

At initial examination, Southern analysis of the δTcR gene on EcoRI digest showed the germline 6.0 Kb band and two additional rearranged bands; the mol-
ecular sizes of these bands were 12.1 Kb and 5.5 Kb, and their intensities, similar to each other, were roughly 30% of that of the germline band (Figure 3); similar results were obtained with Kpn I digest. The analysis of the /H9253 -TcR gene showed reduced intensity of the 1.8 Kb germline band (gene segment J/H9253 1.3) in respect to the other germline band (3.3 Kb, corresponding to the J/H9253 2.3 gene segment), and the presence of a single rearranged band whose intensity was approximately 30% (Figure 3). At variance with the results of the immunophenotypic analysis, the /H9252 -TcR gene also showed a rearranged pattern, compatible with a biallelic rearrangement of the /H9252 2 gene segment; even in this blot, the intensity of the rearranged band indicated that the cells bearing rearrangement of the /H9252 -TcR genes roughly corresponded to 30% of the cells analyzed. These results, together with the data of immunophenotypic analysis, indicated that both CD56+ cell populations had the same genotypic pattern and derived from the same precursor that bore the β-, γ-, and δ-TcR gene rearrangement. A second analysis performed after MACOP-B chemotherapy had been discontinued showed similar results (Figure 3).

In May, during disease progression, a third molecular analysis of the TcR genes was performed. On this occasion the results of the Southern analysis confirmed that clonal cells had completely replaced bone marrow (absence of the germline bands) (data not shown). In addition, the analysis of β-TcR with Hind III digest showed the appearance of an additional faint rearranged band, suggesting the presence of a subclone within the neoplastic cell population.

Forty days later, when the third line therapy had been completed and the patient was in complete remission, Southern analysis was repeated to ascertain at molecular level the status of remission. No rearranged band was detected in any of the experiments performed (Figure 3).

Discussion
The classification of lymphoid neoplasms characterized by NK markers is one of the most debated topics in contemporary hematopathology. The difficulty in distinguishing consistently between NK- and T-cell derived malignancies is not surprising, since in normal tissues these two lineages are closely related, and a bipotential T/NK progenitor cell is presumed to exist. In addition, the attempt to classify these lymphoid disorders can be biased by the fact that often T- and NK-cell-derived neoplasms may display aberrant phenotypes, reflecting either an anomalous genotype or the existence of a rare normal cellular counterpart equipped with an unusual surface membrane mosaic.

The antigen commonly utilized for identifying NK neoplasms and NK-like T-cell malignancies is CD56. Although it has no lineage or disease specificity, its presence is considered to be typical of a large family of NK-related leukemias and lymphomas, including pure NK disorders and T-cell associated diseases. One debated problem regards the possibility of classifying true NK malignancies from NK-like T-cell neoplasms. It is generally retained that the best candidates for true NK neoplasms are tumors characterized by NK-associated antigen expression in the absence of surface CD3 and TcR and with no rearrangement of the TcR genes. Derived from this view is the accepted general rule that NK-like T-cell lymphomas lacking the T-cell antigen CD3 or framework determinants of TcR should exhibit at least TcR gene rearrangement to be distinguished from true NK malignancies.

In this paper we describe a case in which we identified some biological and clinical features consistent with TcR-γδ hepatosplenic lymphoma along with clinico-hematologic characteristics of aggressive NK leukemia. Indeed, at presentation this young adult male showed liver and spleen enlargement with minimal bone marrow involvement; there were signs of hemolytic anemia (low Hb, reticulocytosis, raised unconjugated bilirubin, raised LDH and bone mar-
row erythroid hyperplasia) without detectable anti-erythrocyte antibodies, with spleen enlargement and images of hemophagocytosis in the bone marrow. These are all findings commonly described in patients with TcR-γδ hepatosplenic lymphoma. However, with disease progression all these findings disappeared, and the course became stormy as reported for NK acute leukemia. We consider this case as a unique example of co-existence at diagnosis of the two otherwise distinct above-mentioned diseases. A dramatic change in the relative size of the two populations was observed during the course of the disease: at diagnosis, the CD3+/TcR-δ gene subset was clearly detectable and measurable while during disease progression this cell population virtually disappeared. However, in all disease phases lymphoid cells infiltrating bone marrow showed the same TcR rearranged pattern, consistent with a single progenitor origin. A likely interpretation is that the disease originated from a bipotential T-NK progenitor cell that rearranged the TcR gene cluster but still retained the possibility of differentiating and maturing along a T- or NK pathway, giving rise to two subclones with divergent phenotype and different biological properties. Recently, Ino et al. and Yoshida et al. reported one and three patients, respectively, whose findings support the hypothesis of a putative precursor common to both T- and NK-cells. A few concepts may be inferred from this interpretation: (i) TcR rearrangement does not seem sufficient to produce a T-cell phenotype; (ii) TcR rearrangement precedes the biological switch that determines the final maturation pathway of a T-NK progenitor; (iii) a TcR-rearranged cell may still differentiate towards a true NK-cell.

Isochromosome 7q and trisomy 8 are non-random chromosome abnormalities already associated with γδ T-cell lymphoma. Isochromosome 7q is one of the most commonly occurring isochromosomes in neoplasia. This abnormality may result in both loss and gain of genetic material, thus leading either to loss of a tumor suppressor gene, or to activation of an oncogene, or both. It has been proposed that while (7q) may play a crucial role in the pathogenesis of γδ T-cell lymphoma, trisomy 8 is a secondary event. Indeed, in our patient trisomy 8 was no longer detected in the final phase of the disease. TcR-γδ is located on chromosome 7p, and TcR-β on 7q; an isochromosome 7q may result in the loss of the TcR-γ gene and in the duplication of the TcR-β gene. Ansell et al. suggest that this cytogenetic abnormality in hepatosplenic γδ T-cell lymphoma is indicative of a poor prognosis. Our observation that (7q) was present not only when the patient had a clinical picture and immunophenotype consistent with TcR-δ gene hepatosplenic lymphoma, but also in the advanced phase of the disease with findings typical of aggressive NK leukemia, further supports the hypothesis of a common ancestor for TcR-δ and NK cells.

The two-population pattern and the two-phase course observed in our patient are reminiscent of cases of biphenotypic acute leukemia, in which both myeloid and lymphoid leukemic cells can be observed at diagnosis, carrying the same cytogenetic or molecular marker, and switching to a single component during the course of the disease. Even in this case the most likely explanation is that the oncogenic transformation took place in a cell which was a common ancestor to both lineages. In principle, the phenotypic switch may result from a spontaneous take over of the most aggressive subclone, or it could be favored by the therapeutic intervention, which may differentially affect the two subclones. In our patient the switch occurred before intensive chemotherapy, when only relatively small doses of prednisone had been administered, thus favoring the spontaneous clone replacement hypothesis.


Figure 3. Southern analysis of the joining region of the T cell receptor genes (see text for details of technique and probes used).

Lanes 1: DNA sample from marrow cells at diagnosis. Lanes 2: same, after four weeks of MACOP-B treatment. Lanes 3, 4: DNA extracted from bone marrow and peripheral blood during disease progression after second line treatment failure. Lanes 5: DNA samples at remission after third line chemotherapy. C: control DNA (placental cells); G: germline bands. Arrows: rearranged bands.
very intensive treatment, and final refractoriness to chemotherapy may be attributed to the high gp170 expression on the leukenic cell population, which was not reverted by combining chemotherapy with cyclosporin-A. Indeed, even autologous or allogeneic transplantation has been reported to be ineffective in eradicating this type of disease.24

Contributions and Acknowledgments

AC was the principal investigator and wrote the paper. LP and MRV were responsible for patient care and revised the literature. LL performed flow cytometric analyses. PD and LDV performed and interpreted flow cytometry data. FS and BR supervised the study and gave final approval. The criteria for the authors’ order is: first name: principal investigator and writer; second to eighth name: patient’s care, data handling and interpretation; last names: senior authors, direct supervisors and revisors of the manuscript.

The authors wish to thank Dr. Stefano Pepe who performed the ploidy analysis.

Funding

This work was partially supported by CNR (P. F. Biotechnology), AIRC (Milan), AIL (30 ore per la vita), MURST and Regione Campania, Italy.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

Manuscript received October 21, 1999; accepted February 1, 2000.

Potential implications for clinical practice

An accurate flow cytometry study may identify LGL-derived neoplastic cells even in the absence of a typical morphology.

Although CD3+ and CD3- LGL neoplasms normally present as separate disease entities (i.e., T-lymphoblastic lymphoma and NK-acute leukemia) there are cases of mixed or sequential clinical pictures.

References