Leukemia of Non-T Lineage Natural Killer Cells


An unusual case of an aggressive leukemia of natural killer (NK) cells occurred in a 65-year-old male. Clinical characteristics of this case included hepatosplenomegaly, ascites, marrow infiltrate with leukemic cells, and a WBC up to $82.8 \times 10^9$ before therapy. One year before his presentation he had been noted to have a WBC of $12.1 \times 10^9$ with 78% lymphocytes, and 6 months before had noted intermittent fever and weight loss. He and his brother had well documented hereditary cold urticaria. The patient was treated with a modification of ProMACE CYTABOM regimen and had prompt regression of the leukemia with associated acute tumor lysis. Renal, hepatic, and marrow failure predominated during a terminal course that ended 22 days after therapy was commenced, and at autopsy there was no evidence for leukemic cell infiltrate in the liver, spleen or marrow. The leukemic cells were large granular lymphocytes by light and electron microscopic criteria, and had the following immunophenotype: CD4+, CD3+, CD8+. The cells displayed high antibody-dependent cell-mediated cytotoxicity (ADCC) and NK activity, and had a high rate of spontaneous proliferation in vitro that was not augmented by phytohemagglutinin (PHA), concanavalin A (Con A), or pokeweed mitogen (PWM). Southern analysis of DNA from leukemic cells revealed normal germline arrangements for the $\beta$ and $\gamma$ chains of the T cell antigen receptor and immunoglobulin heavy chain genes. The majority of metaphases were clonally abnormal revealing consistent rearrangements involving extra material attached to the long arms of chromosomes 5 and 11.

ABNORMAL PROLIFERATION of large granular lymphocytes (LGL) has been observed in association with neutropenia, pure red cell aplasia, rheumatoid arthritis, and also as an apparent isolated phenomenon. On the basis of the immune phenotype, a classification has been proposed dividing LGL proliferations into two broad groups: Type A (CD2+, CD3+, CD8+) and Type B (CD2+, CD3-, CD8±). In several instances the clonality of Type A LGL lymphocytosis has been confirmed by analysis of the rearrangement of the $\beta$3 gene, but the disorder has generally not shown a progressive course. Recently a case of LGL leukemia with a clearly malignant course and clonal chromosome abnormalities was described. We report a second instance of LGL leukemia complicating Type B LGL proliferation. Immune phenotyping and $\beta$3 gene arrangement studies indicated a leukemia of non-T lineage natural killer (NK) cells and cytogenetic analysis was suggestive of evolution of the malignant clone.

CASE REPORT

A 65-year-old white man became progressively and insidiously ill with weakness, fatigue, anorexia, and loss of weight, and consulted his physician in May of 1986. Over approximately 6 months he had lost more than 25 pounds and noted intermittent fever. For 1 month he had drenching night sweats. Hepatomegaly, splenomegaly, and generalized wasting were noted. Intermittent fever to 103°F was recorded. His hemoglobin (Hb) was 109 g/L, hematocrit (Hct) 32.3%, platelet count (Plt) 85 $\times 10^9$, and white cell count (WBC) 7.6 $\times 10^9$ with 96% lymphocytes and 4% neutrophils. The serum albumin was 3.4 g/dL, bilirubin 0.76 mg/dL, alkaline phosphatase (ALP) 310 U/L, aspartate aminotransferase (AST) 565 U/L, alanine aminotransferase 660 U/L, and lactate dehydrogenase (LDH) 1,085 U/L. Uric acid was 8.4 mg/dL and creatinine (Cr) 1.0 mg/dL. Serum iron was reduced at 31 mg/dL with elevated total iron-binding capacity (257 mg/dL) and ferritin (2,177 mg/dL). Abdominal computed tomography (CT) scan confirmed hepatosplenomegaly. Evaluation for infectious disease revealed no evidence of infection. He was anergic to tuberculin and mumps antigen. Epstein-Barr virus (EBV) serology showed evidence of past infection. Antinuclear antibodies were not detected and serum immunoglobulins were normal. Morphology and phenotyping of peripheral blood mononuclear cells indicated that the lymphocytosis consisted of LGL and he was referred to Emory University Hospital for evaluation.

In the past the patient had suffered from hereditary cold urticaria, and his brother was the index case of a published family study. The patient was exposed to asbestos dust in his occupation as a foreman for a power utility and previous chest x-rays had shown calcified pleural plaques. He had undergone hemorrhoidectomy and herniorrhaphy in 1961 and transurethral resection of the prostate for benign prostatic hypertrophy together with removal of ureteral calculi in July 1985. A complete blood count (CBC) performed at that time had showed a Hb level of 132 g/L, Hct 36.9%, Plt 155 $\times 10^9$, and WBC 12.1 $\times 10^9$ with 78% lymphocytes. The blood smear was not available for review to determine if the lymphocytes were in fact LGL.

At the time of referral and hospitalization he was jaundiced and the liver and spleen extended 11 and 14 cm below the costal margins, respectively. Ascites was present and the
urine contained bilirubin. No lymph nodes were palpable and signs of liver failure were absent. In 16 days the WBC had risen from 7.6 to 47.5 x 10^6 with 84% LGL. His Hb was 109 g/L with 4.5% reticulocytes (index 1.7) and serum haptoglobin <5 mg/dL. Bone marrow aspirate was hypercellular with normal erythroid, myeloid, and megakaryocytic elements and a lymphoid infiltrate of LGL comprising 49% of the nucleated marrow cells. The trephine biopsy was markedly hypercellular with a diffuse infiltrate of LGL. Abdominal paracentesis revealed sterile ascites with 3.3 x 10^6 WBCs/L, of which 90% were LGL. Liver enzymes and LDH remained markedly elevated, and the bilirubin was 5.3 mg/dL with 57% conjugated. Clotting was abnormal with PT 15.4 seconds, PTT 39 seconds, and fibrinogen 65 mg/dL. The PT and PTT corrected to normal values with addition of normal plasma. The factor VIII coagulant activity was 136% and factor VIII related antigen 576%; fibrin split products were not detected. Repeat CT scan showed progressive splenomegaly and ascites. Night sweats and fever spikes to 104°F continued (Fig 1) and the WBC rose to 82.8 x 10^9 (Fig 2A).

Methylprednisolone was commenced at 2 mg/kg/d on the fifth day in the hospital. The WBC fell to approximately 31 x 10^9 and the fever abated; however, within five days the fever returned, and by seven days the WBC had risen again to 51.8 x 10^9. A single dose of vincristine (2 mg) had no effect. A Hickman catheter was placed for venous access and combination chemotherapy based on ProMACE-CYTABOM regimen was initiated. Following a dose of cyclophosphamide (650 mg/m²) and two doses of etoposide (VP-16) (120 mg/m² per dose), the temperature fell to normal and the WBC to 6.2 x 10^9. This was accompanied by transient elevation of LDH peaking at 4,200, and serum phosphate peaking at 9.3 mg/dL (Fig 2). The WBC and the LDH continued to fall, and the fibrinogen normalized by day 12. The spleen decreased in size to 8 cm palpable without change in liver size. Administration of cytosine arabinoside (Ara-C) (300 mg/m²), bleomycin (5 U/m²), and a further dose of vincristine (2 mg) was followed by a temperature spike to 104.2°F. Multiple blood cultures throughout the period of observation failed to grow pathogens; the clinical course was unaffected by empirical antibiotic therapy. LGL disappeared from the peripheral blood. Renal failure and hyperbilirubinemia developed terminally and he died 22 days after commencing therapy with prednisone.

Autopsy revealed 1.5 L of serosanguinous ascites, a congested liver weighing 2,690 g, a 1,300 g spleen with focal old infarcts, hemorrhagic gastritis, transmural cecal hemorrhages, and acute bronchopneumonia. Histology showed acute renal tubular necrosis and no evidence of infiltration of liver, spleen, or bone marrow with LGL.

METHODS

Morphology, histochemistry, and ultrastructure. Cells from peripheral blood and marrow aspirate were stained with Wright-Giesma stain, periodic acid Schiff reagent, and for acid phosphatase (ACP), alpha-naphthyl acetate esterase (ANAE), and myeloperoxidase activities as previously described. 

Transmission electron microscopy was performed on cells fixed in 1.25% glutaraldehyde in Millonig's buffer, postfixed in 1.25% osmium tetroxide, embedded in Maraglas, cut into thin sections, and stained with uranyl acetate and lead citrate.

Immunophenotyping. For this and other studies peripheral blood mononuclear cells were isolated by Ficoll-Hypaque density gradient centrifugation. Surface antigens were demonstrated by using monoclonal antibodies in an indirect immunofluorescence assay and positive cells were enumerated by flow cytometry.

Functional activity. Antibody-dependent cell-mediated cytotoxicity (ADCC) and NK activities were measured using Cr release assays using murine mastocytoma cells (P815) coated with rabbit IgG (for ADCC) and K562 (for NK) cells as targets and cytotoxicity calculated as previously described.

Proliferation studies. Stimulation with the mitogens phytohemagglutinin (PHA), concanavalin A (Con A), pokeweed mitogen (PWM), and 12-O-tetradecanoyl-phorbol 13-acetate (TPA) was performed as previously described, except that each well of a 96-well
plate contained 2.5 x 10⁶ cells. LGL were also grown in interleukin-2 (IL-2) containing medium as previously described.5

Cytogenetics. Peripheral blood was cultured in RPMI 1640 (Gibco) according to three different protocols: (a) 48-hour culture without stimulation; (b) 72-hour culture with PHA (Wellcome Diagnostics) stimulation; and (c) 72-hour culture with stimulation from IL-2 lymphocyte conditioned media. Cultures were harvested after treatment with colchicin (0.05 µg/mL) (Gibco) for one hour followed by hypotonic treatment with potassium chloride (0.075 mol/L and fixation in cold methanol-glacial acetic acid (3:1). Slides were air-dried and analyzed by Giesma-trypsin-Giesma banding.22 Chromosome abnormalities were designated according to the International System for Human Cytogenetic Nomenclature.23

Tδ, Tγ, and immunoglobulin gene rearrangement. Analyses for T cell antigen receptor gene and immunoglobulin gene rearrangements were performed as previously described.6-8 The rearrangement of the Tδ and immunoglobulin genes of the patient's fresh peripheral blood lymphocytes was compared with the arrangement of these genes in peripheral blood buffy coat cells of normal individuals. High molecular weight DNA samples were extracted from the cells and completely digested with BamHI, EcoRI, and HindIII (International Biotechnologies, Inc, New Haven, CT; and New England Biolabs, Beverly, MA). DNA fragments were size fractionated by electrophoresis on agarose gels, transferred to nitrocellulose paper. The fragments were hybridized to a randomly primed 32P cDNA probe (86TJ) for the constant region of the 1/3 gene,25 a probe to JH region of the immunoglobulin gene (TJy), and a probe to J H region of the constant region of the 1/3 gene,25 washed at the appropriate stringency, and visualized on autoradiograms.

RESULTS

Characterization of LGL. The peripheral blood LGL varied in size and had moderately abundant basophilic cytoplasm with azurophilic granules and frequent cytoplasmic vacuoles (Fig 3). Bone marrow LGL were similar in appearance. The LGL stained with ACP and PAS and were negative for ANAE and peroxidase activity. Transmission electron microscopy (EM) showed prominent cytoplasmic granules with the structure of parallel tubular arrays.

The immunophenotype of Ficoll-Hypaque separated mononuclear cells was CD2+, HLA-DR+, CD3-, CD4-, and CD16+. A minority of cells were positive for Leu7 and NKH1. The small population of CD3+ cells was expanded to 10.5% after 24 hours incubation with IL-2; the proportion of Leu7+ and NKH1+ cells also increased with IL-2 incubation (Table 1).

The LGL displayed high ADCC and NK activity in vitro. NK activity after overnight incubation was 98% to 100% with effector:target ratios (ETR) of 50:1 to 5:1 and was 58% with an ETR of 1:1. Lysis with ADCC ranged from 109% to 97% with ETR's of 50:1 to 5:1 and was 47% with an ETR of 1:1 (Table 2). The LGL had a high spontaneous rate of proliferation in vitro with mean cpm/2.5 x 10⁵ cells of 40,768 ± 3,472 on day 3 of culture. The proliferation was not augmented by PHA, Con A, or PWM (stimulation indices [SI] 0.8 ± 0.1, 0.7 ± 0.1, 0.7 ± 0.1). Very low concentrations of TPA increased day 3 3H-thymidine uptake by 1.6 ± 0.1 and 2.0 ± 0.2 times (1 x 10⁻⁸ mol/L and 1 x 10⁻⁹ mol/L, respectively). After five days of culture, spontaneous

![Fig 3. Wright's stained peripheral blood smear. Lymphocytes have abundant cytoplasm with azurophilic granules and sometimes vacuoles (Original magnification x400, current magnification x200.)](image)

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<th>Table 1. Cell Surface Phenotype Studies</th>
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| **Monoclonal Antibodies** | **Cluster Designation** | **Specificity** | **Percent Positive**
| OKT11 | CD2 | Sheep erythrocyte receptor | 98.5
| OKT3 | CD3 | Mature T cells | 3.5
| Leu1 | CD3 | Pan-T cell | 4.3
| OKT4a | CD4 | T helper/inducer | 3.3
| OKT8 | CD8 | T cytotoxic/suppressor | 1.3
| Anti-HLA-DR | HLA-DR | 96.2
| Leu7 | Large granular lymphocytes | 31.2
| NKH1 | Large granular lymphocytes | 16.4
| Leu11b | CD16 | Fγ receptors | 1.0
| M1 | CD11 | Neutrophils, monocytes, large granular lymphocytes | 7.2

Table 2. In Vitro Cytotoxicity

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<th>Effector:Target Ratio</th>
<th>NK Activity</th>
<th>ADCC</th>
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<tr>
<td>50:1</td>
<td>98</td>
<td>109</td>
</tr>
<tr>
<td>25:1</td>
<td>92</td>
<td>104</td>
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<td>100</td>
<td>97</td>
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<tr>
<td>1:1</td>
<td>58</td>
<td>47</td>
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Percent cytotoxicity was calculated according to the formula: 100 x cpm [test wells] - cpm [spontaneous release]/cpm [maximum release] - cpm [spontaneous release].

*Target cell K562.
†Numbers represent the mean of two experiments.
‡Target cell P815.
proliferation had declined, with mean cpm/2.5 × 10^5 cells of 11,824 ± 517. Day 5 ^3H-thymidine uptake was stimulated by PHA (SI 9.7 ± 0.1). Intermediate concentrations of TPA had a suppressive effect.

**Genetic analysis of LGL.** Based on southern analysis of DNA from the leukemia cells, the LGL were found to retain the germline configuration for the β and γ T cell antigen receptor and the immunoglobulin heavy chain gene. The Southern blots using a c-DNA probe for the constant region of the T-β chain are displayed in Fig 4.

Cultured lymphocytes from both unstimulated and PHA stimulated growth conditions gave mixtures of normal (25%) and clonally abnormal (75%) metaphases. In contrast, all metaphases observed from IL-2-stimulated cultures were cytogenetically abnormal. Twenty-one abnormal cells were fully karyotyped, revealing consistent rearrangements involving extra material attached to the long arm of chromosomes 5 and 11 creating derivative chromosomes der(5) and der(11) (Fig 5). The banding pattern of the involved region of chromosome 5 suggests its origin may be from a terminal duplication of 5q. The limited amount of extra material on chromosome 11 precluded identification of its origin. Three of the abnormal metaphases also showed extra material attached to the short arm of chromosome 6. This material lacks distinct bands and therefore resembles a homogeneously staining region.

**DISCUSSION**

The lymphoproliferative disease affecting this patient was clearly derived from a cell population of LGL on the basis of typical morphology and histochemistry. The lack of expression of the T cell receptor antigen CD3 and the germline state of the Tβ and Tγ genes differ from the CD3+, Tβ+ phenotype of most cases of large granular lymphocytosis studied and places this instance of LGL proliferation into the Type B category. The lymphocytosis was a clonal expansion on the basis of karyotypic abnormalities involving chromosome 5 and 11 with evidence of further evolution of chromosomal abnormalities in subpopulations of the cells. Such a pattern is consistent with the development of an aggressive malignant phase after a period of asymptomatic monoclonal natural killer cell lymphocytosis. This progression of the disorder bears similarities to that seen in the proportion of cases of B-cell chronic lymphocytic leukemia.

![Fig 4. Radioautograph of Southern blot to define the T-cell receptor β (Tcr-β) gene arrangement. (A) EcoRI digest of Epstein Barr virus B-cell line (lane a), patient (lane b), clonal T cell (lane c), and polyclonal T cell (lane d). The pattern in patient DNA is comparable to germ line. (B) HindIII digest of Epstein Barr virus B-cell line (lane a), patient cells (lane b), clonal T cells (lane c), and polyclonal T cells (lane d). The insert demonstrates the positions of the variable, diversity, joining the constant Tcr-β genes indicated by V, D, J, and C, respectively. The sites of the EcoRI, HindIII, and BamHI digests are indicated by RHB, respectively.](image)

![Fig 5. Partial karyotype from two different metaphases from LGL culture showing clonally abnormal chromosome (right) and normal homolog (left). Extra material of unknown chromosomal origin is attached to the short arm of chromosome 6, and the long arm of chromosomes 5 and 11 creating derivative chromosomes. All 21 metaphases examined from cultures stimulated with IL-2 demonstrated the der(5) and der(11). Three metaphases also showed the der(6).](image)
which evolves into aggressive malignant lymphoma (Richer's syndrome) and to the blast crisis of chronic granulocytic leukemia.

The characteristic clinical manifestations of Type A LGL disease include neutropenia or other cytopenias often complicated by bacterial infections, in association with rheumatoid arthritis, seropositivity for rheumatoid factor of other evidence of non-organ-specific autoimmune disease. The small number of patients with Type B LGL disease so far reported precludes generalization regarding clinical characteristics. Although a number have been asymptomatic, our patient and that of Fernandez et al both died as a result of a clinically malignant process associated with clonal cytophenic abnormalities.

Signs of a progressive lymphoproliferative disease involving blood, bone marrow, spleen and liver were present and associated with fever, drenching sweats, wasting, anemia, thrombocytopenia, significant liver dysfunction and laboratory evidence of coagulopathy. The high spiking fever and sweats were prominent features of the clinical course and could not be ascribed to infection. NK cells are known to elaborate IL-1 and LGL from patients with LGL disease have been shown to secrete lymphokines in vitro. The fever may have been due to in vivo production of IL-1, TNFα, or IL-6 (endogenous pyrogen) by the malignant cells have been shown to secrete lymphokines in vitro. The fever may have been due to vivo production of IL-1, TNFα, or IL-6 (endogenous pyrogen) by the malignant cells or reactive normal cells, and the anorexia and weight loss might also have been the result of lymphokine secretion. The anemia did not appear to be due to marrow replacement by LGL; erythroid precursors were plentiful in marrow aspirate and trephine biopsy specimens. Evidence of Coombs-negative hemolysis was present with continued reticulocytosis and low serum haptoglobin levels and was probably a result of hypersplenism from LGL infiltration in the spleen and portal hypertension. No evidence of pathologic erythroagglutination was present in the bone marrow aspirate. The hypersplenism may also explain the moderate thrombocytopenia with plentiful megakaryocytes in the marrow. Progressive liver dysfunction was a major component of the clinical picture and was characterized by hepatomegaly, jaundice, ascites, hypoalbuminemia, hyperbilirubinemia, and elevated ALP, AST and ALT. In addition, a coagulopathy was present with prolonged screening tests, very low fibrinogen and elevated factor VIII coagulant and factor VIII VWF levels. Circulating anticoagulant and fibrin/fibrinogen split products were absent. These features suggest that the coagulopathy was due to underproduction of coagulation factors by the liver. Cleared view of disseminated intravascular coagulation was lacking.

We elected to take a conservative therapeutic approach initially in the hope that the disease would respond to corticosteroid therapy. Although the WBC and LDH fell and the temperature returned to normal, the response was short-lived and after a few days the WBC and LDH were again rising and the fever returned. Vincristine as single agent had no effect. In an attempt to induce a remission, we commenced combination chemotherapy with modified ProMACE-CYTA-BOM (doxorubicin and methotrexate were deleted because of poor liver and renal function and the dose of etoposide [VP-16] increased). A dramatic clinical and laboratory response followed administration of etoposide and cyclophosphamide with a fall of WBC to normal range, normalization of temperature and evidence of tumor lysis syndrome (rise in phosphate, uric acid, and LDH) accompanied by improvement in liver function with fall in ALP, AST, and bilirubin levels and normalization of serum fibrinogen level. In addition, the spleen and liver diminished in size. Administration of Ara-C, bleomycin, and vincristine was followed by elimination of LGL from the peripheral blood.

After this initial improvement, the patient developed progressive hyperbilirubinemia and renal failure in the setting of chemotherapy-induced neutropenia and died with bronchopneumonia. Although no pathogens were isolated during life, Candida tropicalis was cultured from postmortem blood and lung samples and death was probably caused by systemic fungal infection in a host immunocompromised from cachexia, defects in cell-mediated immunity (cutaneous anergy), and chemotherapy-induced neutropenia. The sensitivity of the malignant NK cells to cytotoxic chemotherapy was confirmed by the absence of LGL from histologic sections of autopsy specimens of bone marrow, liver, and spleen.

The leukemia in this case and in that of Fernandez et al represents the malignant transformation of cells bearing the morphologic, immunologic (with the exception of diminished expression of Leu 7 and M1 antigens in our case) and functional phenotype of typical mature adult peripheral blood LGL. The current case is the first example in which Type B LGL disease with known germline Tβ and Tγ genes has been proven to be clonal. The phenotype of the cells involved in the more common Type A LGL proliferation correlates to that of normal neonatal peripheral blood and adult bone marrow LGL. Although monoclonality in Type A LGL disease has been documented by clonal Tβ gene rearrangements in the majority of cases reported, clear clinical evidence of malignancy has been lacking and in some cases the lymphocytosis has even resolved spontaneously. The existence of a leukemia of LGL of mature phenotype and possessing (unstimulated) NK and ADCC functional activity in vitro but lacking Tβ gene rearrangement is supportive evidence of a pathway of NK cell ontogeny separate from T-cell development. Similar observations have been made in the murine SCID model in which all immunoglobulin and T-cell antigen receptor gene recombinations appear defective, yet normal NK cells are present. We postulate that CD3-, Tβ-, Tγ-, LGL represent a phylogenetically more primitive cell population that retains the capacity for nonspecific cellular cytotoxicity and that the CD3+, Tβ+ LGL are a subset of the phylogenetically more advanced T cells in which cellular cytotoxicity has become an antigen-specific function by virtue of the development of the T cell antigen receptor complex. As a result of the information obtained from Tβ gene rearrangement studies in patients with LGL diseases, including this case, the differentiation model for Leu 7+ cells proposed by Abo et al can now be revised along the following lines. Cells destined to become T3+ LGL arise from fetal Leu 7+, CD3−, CD8−, CD4+, CD11−, HLA-DR cells, undergo Tβ gene rearrangement in the fetal liver or bone marrow and
fetal or adult thymus, express CD3, CD8, and CD4 concurrently during their thymic development, and are then exported to bone marrow, spleen and lymph nodes as mature Leu 7 +, CD3 +, CD8 +, CD4 –, CD11 +, HLA-DR thymus-derived antigen-specific LGL. Cells destined to become CD3 – LGL also arise from fetal cells expressing Leu7 and mature to become Leu7 +, CD3 –, CD8 +, CD4 +, CD11 + thymus-independent NK cells. Mature cells in both lineages express CD23; expression of HLA-DR is variable [10] and may depend on activation status and microenvironmental location (spleen, liver, bone marrow, lymph nodes, blood). The available data do not permit a firm designation of the stem cell for CD3 – LGL being a myeloid cell, a lymphoid cell, or a totipotential stem cell.

ACKNOWLEDGMENT

We acknowledge the skilful secretarial assistance of Elizabeth Gregory and Julie Cook, and the contributions of Dr Carlos Osmon, the referring physician.

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