Aggressive Natural Killer Cell Leukemia in an Adult With Establishment of an NK Cell Line

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There have been many reports of cases in which chronic increases in the numbers of natural killer (NK) cells have been reported. Whether this is reactive or neoplastic in nature has been debated. We report the first case of an aggressive NK cell leukemia in an adult with establishment of an NK cell line. A 70-year-old man had two spontaneous episodes of jejunal perforation and one month later developed a severe febrile illness with moderate splenomegaly. Hemoglobin was 13.1 g/L, and WBC count was 1.8 x 10^9/L with 2% large granular lymphocytes (LGLs). Platelet count was 143 x 10^9/L protonberbin time (PT) and partial thromboplastin time (PTT) were normal. Bone marrow was infiltrated with 25% to 30% LGLs; serum lysozyme was normal. Serum LDH was initially 1,191 U/L and rose to 6,408 (normal 240 to 525 U/L). Ten days later, the WBC count increased to 99.9 x 10^9/L with 70% LGL cells; the PT and PTT increased, and the platelet count dropped. No bacterial or viral cause of fever was identified. The cells from peripheral blood were LGLs that stained positively for acid phosphatase. All of the LGLs reacted with a monoclonal antibody reactive with NK cells (LEU-11b). Functionally, the patient’s peripheral blood mononuclear cells (PBMs) demonstrated 100 times more lytic activity against K562 tumor cell lines than did normal PBMs. The patient’s PBMs were propagated in vitro. The cultured cells showed the morphological, cytochemical, immunological, and functional characteristics of NK cells. In addition, partial trisomy involving chromosome 1q with duplication in regions of q21 through q31 was observed in all metaphases analyzed. The extra chromosome 1q with duplication in regions q21 through q31 was translocated to the p-terminal of chromosome 5. One percent to 5% of normal PBMs comprise NK cells; in most cases, leukemias arise from normal phenotypic counterparts. This case demonstrated that aggressive NK cell leukemia may occur in adults. In addition, the chromosomal abnormalities suggest that this is not a reactive process but a malignancy.

GREAT HETEROGENEITY exists among human mononuclear cells, yet in almost all cases of leukemia, normal counterparts for the phenotypic patterns of leukemia cells have been identified. The natural killer (NK) cell is one such cell type. Normally, NK cells comprise ~3.6% of peripheral blood mononuclear cells and are also present in the bone marrow and spleen. Morphologically, NK cells are large granular lymphocytes (LGLs), with cytoplasmic granules that stain positively for acid phosphatase and react with monoclonal anti LEU-11b. The cells are identified functionally by their ability to lyse specific targets, such as the K562 tumor cell. Numerous case reports have described patients with long-standing increases in the numbers of NK cells; whether this increase is reactive or neoplastic in nature has been debated. In addition, a few cases of NK cell leukemia in children from whom cell lines have been successfully established have been described. We believe that this is the first report of an aggressive NK cell leukemia in an adult with the establishment of a leukemia cell line that retained the morphological, immunologic, and functional characteristics of NK cells.

Most lymphomas initially exhibit infiltration of organs by a particular mononuclear cell type. In many such cases, the mononuclear cell may suddenly increase in the peripheral blood and a leukemic state is heralded. We have observed a case in which the particular cell type was identified as a NK cell. Initially, leukemic cells were present in the bone marrow, spleen, and the lamina propria of the jejunum. Within ten days, the functionally active NK cells increased in the peripheral blood. The patient became febrile, developed a bleeding diathesis, and died.

MATERIALS AND METHODS

Patient. A 71-year-old white man who had an acute abdomen was found to have a jejunal perforation. At this time, an incidental abdominal aneurysm was noted. One month later, he again developed jejunal perforation. Two months later, he was admitted to the surgical ward for repair of his aortic aneurysm. He was febrile, with a temperature of 39 °C. He had minimal cervical lymphadenopathy and moderate splenomegaly. His hemoglobin was 13.1 g/L, hematocrit 404, WBC count 1.8 x 10^9/L with 1% metamyelocytes, 6% bands, 78% polymorphonuclear cells (PMNs), 13% lymphocytes, and 2% cells that had a morphology resembling LGLs. Platelet count was 143 x 10^9/L, prothrombin time (PT) was 11 seconds, and partial thromboplastin time (PTT) was 32.2 seconds. A bone marrow aspirate showed dysplastic erythropoiesis, but was infiltrated with 25% to 30% cells that had the morphology of LGLs. Serum lysozyme was 5.7 μg/mL (normal 3.3 to 6.6 μg/mL). Serum lactic dehydrogenase (LDH) initially was 1,191; later in the course of the disease, it rose to 6,408 U/L (normal 240 to 525 U/L). EKG and chest x-ray were normal. The ultrasound showed no intraabdominal collection of fluid. Liver and spleen scans showed a normal liver and an enlarged spleen. Protein electrophoresis was relatively normal, with IgG 13.42 g/L, IgA 4.79 g/L, and IgM 0.4 g/L. Antinuclear antibody and anti-DNA was within normal limits. Viral studies, which included hepatitis antigen and antibody, hepatitis surface antigen, toxoplasma, and EBV, were negative. Blood cultures for aerobic and anaerobic organisms were negative. Because the patient was relatively neutropenic, febrile, and constitutionally ill, he was treated empirically with carbencillin, tobramycin, metronidazole, and prednisone.

One day after admission, he developed bleeding from his gastrointestinal tract. His platelet count was 15 x 10^9/L. Both PT and PTT were more than two minutes. White cell count was 35.4 x 10^9/L, with 50% large granular LGLs. At gastroscopy, multiple gastric ulcers ranging in size from 0.5 to 2 cm in diameter were seen. There was nodularity of the mucosa. He was treated symptomatically with H2 blockers, transfusion of fresh frozen plasma, and cryoprecipitate.
without any change in the bleeding diathesis. Very rapidly, he developed generalized ecchymosis and died. Just before death, his white count had risen to 99.9 x 10^9/L, with 70% LGLs. Autopsy showed that he had infiltration of LGLs in the spleen, lymph nodes, and bone marrow. Autolytic changes made interpretation of the exact nature of infiltrating cells in the lamina propria and submucosa of the stomach and small bowel difficult. However, review of small bowel removed at initial surgery for perforation revealed that cells that were compatible with the morphology of LGLs were infiltrating the lamina propria.

**Morphology of cells.** Peripheral blood and bone marrow slides and the cultured NK cell line were stained routinely with Wright's-Giemsa, peroxidase, Sudan Black B, periodic acid-Schiff (PAS), specific and nonspecific esterase and acid phosphatase. In addition, suspensions of PBMs and the cultured NK cell line were stained with fluorescein-conjugated monoclonal antibodies to OKT3, OKT4, OKT8, and LEU-11b antigens. In brief, 5 μL of the reconstituted monoclonal antibody to OKT3, OKT4, OKT8 and LEU-11b antigens was added to 200 μL of cell suspension containing RPMI 1640 and 5% fetal calf serum (FCS). The cell suspension was mixed, placed on ice for 30 minutes, and mixed every ten minutes. The cell suspension was then washed twice with cold RPMI 1640 and resuspended in 100 μL of RPMI 1640. To this suspension was added 100 μL of fluorescein-conjugated goat anti-mouse antibody in 1:20 dilution. The suspension was mixed, placed on ice for 30 minutes, and mixed every ten minutes; it was then washed three times with cold phosphate-buffered saline (PBS). Two to three drops of buffered glycerol saline were added to the cell button, mixed and placed on a glass slide, and covered with a cover slip. Cells with fluorescence were enumerated by a Zeiss ultra violet microscope with a vertical illuminator.

**In vitro establishment of NK cells.** Heparinized blood from normal individuals or from our patient was diluted 1:1 with PBS and centrifuged for 30 minutes at 400 g on Ficoll-Hyapaque (Pharmacia, Piscataway, NJ). The mononuclear cell fraction remaining at the interface was washed with PBS and resuspended in complete medium [RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (GIBCO, Grand Island, NY), 100 IU/mL of penicillin, 100 μg/mL of streptomycin, 2 mmol/L of glutamine, and 5 x 10^-5 mol/L of 2-mercaptoethanol].

Mononuclear cells from the patient were cultured in 25-cm² flasks (Falcon 3021, Falcon, Oxnard, Calif.) in 10 mL of complete medium at an initial concentration of 1 x 10^6 cells per milliliter. In some of the cultures, the medium was supplemented with 20% T cell growth factor (TCGF). The TCGF was cultured from the cell-free supernatant of cultured rat spleen cells which had been pulsed for two hours with 10 μg/mL Concanavalin A, washed three times with PBS, and incubated for 24 hours at 5 x 10^6 cells/mL in complete medium. In all cases, approximately two-thirds of the medium was replaced in the cultures three times per week. To maintain cell numbers of ~10^6 cells per milliliter, cultures were subdivided when sufficient growth occurred.

**Estimation of cytotoxic activity.** Cytotoxicity was estimated by chromium release assay. Approximately 2 x 10^6 K562 target cells were suspended in 0.2 mL of PBS and incubated for one hour at 37°C with 100 μCi ⁵¹Cr (NEN, Lachine, Quebec). Target cells were washed three times with PBS and resuspended in complete medium at 1 x 10^6 cells per milliliter. Target cells were pipetted in 0.1 mL vol into 96-well tissue culture multiwells (#76-013-05, Flow Laboratories, McLean, Va) to give 10⁴ cells per well. The effector cells were suspended in complete medium at a range of concentrations and were pipetted into the trays in 0.1-mL vol to give a range of effector-target (ET) ratios. The trays were centrifuged for five minutes at 40 g, incubated for four hours at 37°C in 5% CO₂, and centrifuged for ten minutes at 700 g. The supernatant was removed from the cells, and 100-μL samples were counted in a Beckman Gamma 8000 counter. Maximum release was estimated as cpm from samples containing 1% Triton X-100. Minimum release was estimated in cultures containing medium alone. The percentage of lysis of the target was calculated as: sample CPM - minimum CPM/maximum CPM - minimum CPM x 100.

**Chromosome analysis.** Cells cultured in TCGF-supplemented medium were harvested on the tenth day. Metaphase spreads were prepared by the conventional air-dry technique and stained according to G-banding procedures.:

**RESULTS**

**Characterization of LGLs from peripheral blood, bone marrow, and established cell line.** Peripheral blood LGLs were variable in size. Most of the LGLs had a low nuclear-cytoplasmic ratio and intense basophilic cytoplasm that contained prominent azurophilic granules. Relatively dense chromatin and nuclei were observed in most of these cases. Auer rods were absent, and rare vacuoles were detected (Fig 1). LGLs stained positively for both acid phosphatase and nonspecific esterase. LGLs did not express OKT3, OKT4, or OKT8 surface antigens. However, 100% of these cells stained with LEU 11b monoclonal antibody.

The cellularity of the bone marrow was moderately increased, with megaloblastic and dysplastic erythropoiesis. Granulopoiesis and megalakaryocytopenia generally appeared normal. The plasmacytoid mononuclear cells constituted 25% to 30% of all nucleated cells in the marrow. The morphology was the same as that of those seen in the peripheral blood (Fig 2). Cytological reactions were not conclusive; however, the LGLs stained positively for both acid phosphatase (Fig 3) and nonspecific esterase.

The cells from the established cell line had morphology and cytochemistry similar to that of the peripheral blood and bone marrow LGLs. In addition, the established cell line did not stain with OKT3, OKT4 or OKT8 antibodies. However, all of these cells stained with LEU 11b monoclonal antibody, similar to the immunologic characteristics of the peripheral blood LGL.

**Growth of NK cells.** PBMs from our patient maintained viability in culture for two weeks. During this time, there was no apparent difference between cultures maintained with or without growth factors. However, in the third week the cells entered a "crisis period." Cells cultured without TCGF died.

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**Fig 1. Large granular lymphocytes in peripheral blood.**
and cells cultured with TCGF did not proliferate. After an additional two weeks of maintenance, cells cultured in TCGF began to proliferate and were maintained for an additional two months in culture. Sufficient growth was obtained to subculture twice a week (1:3 split).

**Cytotoxic assays.** Table 1 shows the results of assays of the cytotoxic activity of fresh PBMs from three normal individuals and from the patient. The cytotoxic activity of PBMs of the patient was considerably higher than that seen in normal individuals. The activity of fresh PBMs from the patient at an ET ratio of 1:1 was equivalent to that seen at a ratio of 100:1 in some normal individuals. In addition, this ability to lyse K562 target cells was maintained when the patient’s PBMs were cultured, and good lytic activity was seen after 60 days in culture with TCGF.

**Chromosomal analysis.** Twenty cells were analyzed. Partial trisomy involving chromosome 1q with duplication in regions q21 through q31 was observed in all 20 metaphases. The extra chromosome 1q with duplications in regions q21 through q31 was translocated to the p-terminal of chromosome 5 (Fig 4). The absence of the Y chromosome in 9 of 20 cells (45%) analyzed was also significant.

**DISCUSSION**

In this article, we provide evidence in an adult for a case of highly aggressive leukemia with the morphology and functional capability of NK cells. This conclusion was based on the finding, in the patient’s blood and marrow, of abnormally high numbers of LEU-11b, T3+, T4−, T8+. LGLs that stained positively for acid phosphatase. The conclusion that the leukemia cells were NK cells was strengthened by the finding that the patient’s PBMs had a greatly enhanced ability to lyse K562 target cells. That these abnormal LGLs were malignant, rather than the result of an abnormal proliferation of NK cells, was based on the finding of chromosomal abnormalities in a cell line established from the patient’s peripheral blood.

In normal individuals, most NK cells are present in the peripheral blood, bone marrow, and spleen. In contrast, NK cells are rare in the lymph nodes. In animals, NK cells have also been found in the lamina propria of the small bowel.16 Our patient initially developed jejunal perforation in a location where NK cells have been found experimentally. In addition, he had splenomegaly without lymphadenopathy; 30% of his marrow cells and, ultimately, 70% of his PBMs were LGLs.

The patient was mildly granulocytic. The cause is unknown; however, this effect may have been due to the suppressive effect of the leukemia cells. It is known that NK cells suppress granulopoiesis in vitro.17 Most patients with chronic NK cell leukemia develop severe neutropenia, recurrent infection, and hypogammaglobulinemia. However, our patient had neutropenia without evidence of recurrent infection.

The patient developed marked swings in temperature, but no cause of infection could be found. It is now known that NK cells produce Interleukin-1, which is analogous to endogenous pyrogen.18 Thus, the febrile response may have been due to mediator release by the NK cells, although a viral cause cannot be excluded since enhanced NK cell activity had been observed during several acute viral infections.19,20 Although the rise in NK cells in our patient may have been secondary to a viral infection, we believe that this is unlikely because of the observed combination of high levels of LGLs.

**Table 1. Cytotoxic Activity of NK Cells**

<table>
<thead>
<tr>
<th>Sample Tested</th>
<th>Source of Cells*</th>
<th>100:1</th>
<th>25:1</th>
<th>4:1</th>
<th>1:1</th>
<th>.25:1</th>
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<tr>
<td>Normal 1 PBMs</td>
<td></td>
<td>48.5</td>
<td>27.4</td>
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<td>-</td>
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<tr>
<td>Normal 2 PBMs</td>
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<td>46.6</td>
<td>13.6</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Normal 3 PBMs</td>
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<td>19.8</td>
<td>8.2</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Patient PBMs</td>
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<td>90.1</td>
<td>89.6</td>
<td>63.1</td>
<td>35.5</td>
<td>11.6</td>
</tr>
<tr>
<td>9-Day culture (no TCGF)</td>
<td></td>
<td>88.4</td>
<td>86.5</td>
<td>63.8</td>
<td>26.5</td>
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<tr>
<td>9-Day culture (TCGF)</td>
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<td>85.4</td>
<td>85.5</td>
<td>54.1</td>
<td>21.7</td>
<td>-</td>
</tr>
<tr>
<td>35-Day culture (TCGF)</td>
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<td>-</td>
<td>-</td>
<td>77.3</td>
<td>45.7</td>
<td>20.5</td>
</tr>
<tr>
<td>60-Day culture (TCGF)</td>
<td></td>
<td>-</td>
<td>95.2</td>
<td>43.8</td>
<td>17.8</td>
<td>-</td>
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</table>

*Fresh peripheral blood mononuclear cells (PBM) or PBMs from the NK cell leukemia patient cultured with or without growth factor (TCGF) for varying lengths of time.

†Varying numbers of effector cells were mixed with $10^6$ K562 target cells to give the effector-target (ET) ratios.
Fig 4. Partial karyotypes of three metaphases showing the marker chromosome (M), a pair of normal chromosomes (1) and a normal chromosome 5. The marker chromosome consists of chromosome 1q with duplication in bands q21 through q31 (bracketed), translocated to the terminal region of the p-arm of chromosome 5. The centromere of each chromosome is indicated by a horizontal line. To compare the band pattern of the extra chromosomal material with the normal chromosomes 1q, the centromeres of the marker chromosome are not aligned with the centromeres of the normal chromosomes.

in the patient’s blood and marrow, enhanced lytic activity, and chromosomal abnormalities.

The coagulopathy that developed was suggestive of disseminated intravascular coagulation (DIC). DIC has been reported to occur in many malignant states, including leukemias, and is most common in acute promyelocytic leukemia. However, in a few reported cases of increased NK cell activity representing either leukemia or lymphoma, DIC has not been reported. Most cases of NK cell leukemia in adults are chronic in nature, and the disease is generally not aggressive. Our case differed in its acute aggressive clinical course; in addition, our case did not express T cell antigens.

Cells with LGL morphology, with or without cytotoxic activity, have been described in patients with lymphoproliferative disorders, in adults with chronic leukemia, and in children with acute leukemias. In some but not all cases, LGL morphology has been associated with the functional ability of the cells to lyse or bind to K562 target cells. In one case of acute leukemia, lytic activity was seen only after the incubation of the PBMs for 24 hours at 37 °C. The PBMs from our patient not only had a high proportion of LGLs, but also displayed high, spontaneous cytotoxic activity at very low ET ratios. These results contrast markedly with those seen with PBMs from patients with lymphocytic leukemias of non-LGL morphology, in which NK cell activity has frequently been found to be depressed as compared with that of normal subjects.

Cell lines established from a child with lymphoblastic leukemia with NK activity have been previously reported. These cell lines retained the surface markers and morphology of the leukemia cells, but lost much of their functional capability, having less lytic activity than that of normal PBMs. In our case, the established cell line retained the morphology, surface markers, and high cytotoxic activity of the leukemia cells seen in the fresh sample of blood from our patient. However, in both cases, the established cell lines were negative for T cell-associated surface markers and were positive for NK cell markers. The cell lines from the childhood leukemia case were negative for T3, T4, and T8, but were positive for Asialo-GM1. Both the fresh blood cells and the cell line from our patient were also negative for T3, T4, and T8, but were positive for the NK-associated marker, LEU-11b. This pattern of expression of surface markers is markedly different from that seen with most adult chronic leukemias and lymphoproliferative disorders expressing LGL morphology and NK cell activity. These cells are uniformly positive for T3 and are frequently positive for T4 and/or T8. In addition, it is unlikely that the established cell line was an NK-like cell derived from a normal cell component of the patient’s blood as has been reported by Brooks et al. NK-like clones have been obtained from various sources including cultured cells, mixed leukocyte reactions, and cytotoxic T cell lines. However, such cell lines invariably expressed T cell-associated surface markers, even when the precursor cells were depleted of T cells prior to culture. Because our cell line never expressed T cell markers, and because the functional capability of the cells did not alter upon culture, it is most likely that the cultured cells were derived from the leukemic LGLs in the patient’s blood. Essentially all human NK cell activity is mediated by LEU-11+ and LEU-7+ cells although LEU-11+, LEU-7+ cells have the most potent lytic activity and LEU-11+, LEU-7+ cells have the weakest activity on a per cell basis. The very potent activity seen with the cells of our patient was associated with LEU-11+ cells.

Cytogenetic analysis of the cell line provided evidence that
the LGL proliferation seen in this patient was a neoplastic rather than a reactive process. Cytogenetic analysis showed the presence of an extra chromosome, 1q, with duplication in regions q21 through q31 in all 20 metaphases analyzed. Although chromosomal abnormalities have been reported to arise spontaneously in vitro, they usually occur after 18 to 20 months of in vitro cell culture. In our case, a 100% abnormal cell expansion of metaphases analyzed could not have arisen within ten days of culture before the cell crises occurred. Therefore, the established NK cell line was most likely derived from the leukemic NK cells in the patient. Clonal chromosomal abnormality involving the acquisition of an extra chromosome 8 or 14 was recently reported in two cases of chronic NK cell leukemia. Our study documents clonal proliferation in a case of aggressive NK cell leukemia.

Partial trisomy involving the whole or part of the q-arm of chromosome 1, and in particular the duplication of regions q21 through q31, has been reported in a large variety of peripheral blood malignancies as well as in solid tumors. Although an etiological role of this type of defect in human cancer cannot be ruled out completely, the general consensus was that abnormality involving chromosome 1q was a secondary event that was selected because it was able to confer proliferative advantage to the existing tumor cells. Additional karyotypic changes involving the loss of a sex chromosome were observed in ~50% of myeloproliferative disorders. Patients who had this additional abnormality seemed to have a poorer prognosis. Almost half of the NK tumor-cloned cell population in our patient had also lost a Y chromosome.

In summary, our case demonstrates that aggressive NK cell leukemia (T3+, T8−, T4− and LEU 11b+) can develop in adults, probably arising from its normal phenotypic counterpart. These cells were established in vitro and retained their morphological, immunologic, and functional characteristics. The presence of chromosomal abnormalities suggests that this was not a reactive process but a malignancy.

ACKNOWLEDGMENT

The authors thank Miss Pat Godin for secretarial assistance.

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