Induction of NK Activity in Large Granular Lymphocyte Leukemia: Activation With Anti-CD3 Monoclonal Antibody and Interleukin 2

By Thomas P. Loughran, Jr, Kevin E. Draves, Gordon Starkebaum, Pamela Kidd, and Edward A. Clark

Large granular lymphocyte (LGL) leukemia is a rare disease characterized by clonal expansion of LGL associated with chronic neutropenia, multiple auto-antibodies, and occasionally polyarthritits. We studied cell surface antigen expression and functional activity of leukemic LGL from ten such patients. Using two-color flow cytometric analysis, we found that leukemic LGL from all ten patients expressed CD3 and HNK-1 markers, while cells from only four patients expressed IgG Fc receptors (FcR). The LGL leukemic cells had little or no NK activity (defined as MHC-nonrestricted cytotoxicity against K562 target cells); however, NK activity could be induced in leukemic LGL by in vitro treatment with as little as 0.05 µg/mL of anti-CD3 monoclonal antibody. Cell sorting experiments demonstrated that NK activity was induced in CD3+ leukemic LGL (either CD3+, HNK-1+ or CD3+, FcR+) with anti-CD3 monoclonal antibody but not in normal CD3+ or FcR- T cells. Treatment with purified interleukin 2 (IL 2) also caused direct activation of some CD3+ leukemic LGL. Despite induction with anti-CD3 MAb or IL 2, activated leukemic LGL did not proliferate or express high density IL 2 receptors detectable by cell sorter analysis. Treatment with alpha interferon had minimal effect on NK activity of LGL leukemic cells. These results suggest that leukemic LGL may provide a useful model for examining the signals required for LGL maturation and activation.

METHODS

Patients. Patients were referred for evaluation of chronic lymphocytosis or neutropenia; three patients had polyarthritis, five had recurrent infections. Clinical details of patients 1, 6, 9, and 10 have been previously described. Examination of Wright-Giemsa stained blood smears showed that all patients had markedly increased numbers of LGL (LGL counts ranged from 1,700 to 20,000 cells/mm²; normal values in our laboratory: 223 ± 99 cells/mm², n = 10). Chronic neutropenia (< 1,800 neutrophils/mm²) was present in all patients with the exception of patient 5 who had a normal neutrophil count. Patient 2 had acquired cyclic neutropenia. All patients were studied on protocols approved by the Human Subjects Review Committee of Fred Hutchinson Cancer Research Center or the University of Washington.

Lymphocyte phenotyping. Peripheral blood or splenic mononuclear cells were isolated by Ficoll-Hypaque (Pharmacia, Piscataway, NJ) density gradient centrifugation and studied for expression of cell surface antigens using an EPICS 5 flow cytometer (Coulter Corporation, Hialeah, Fla) with direct one-color and two-color analysis using a panel of fluorescein-conjugated or R-phycoerythrin (PE)-conjugated mouse MAb, as previously described. Normal human splenic tissue was obtained at time of harvesting of cadaveric kidneys for renal transplantation. The antigens recognized by the MAb are described using the current World Health Organization approved international nomenclature. The following MAb have been verified by international workshop analyses: Dr Jeff Ledbetter (Oncogen Corporation, Seattle) kindly provided the G19-4 anti-CD3(T3), G17-2 anti-CD4(T4), and G10-1 anti-CD8(T8) antibodies; Dr John Hansen (Fred Hutchinson Cancer Research Center, Seattle) kindly provided the 9.6 anti-CD2 (E-rosette receptor) and the 60.3 antibody to the Lp95-150 (LFA-1 associated) complex. Dr Toru Abo (University of Alabama, Birmingham, Ala) provided the HNK-1. The HB10a MAb reacts with a major histocompatibility complex monomorphic determinant on Class II beta chains while the FC-1 MAb recognizes IgG Fc receptors (FcR; CD16) found on NK cells. Competitive binding experiments show that FC1 recognizes an FcR epitope also recognized by Leu-11. The JAC5 MAb recognizes a 220,000 dalton polypeptide (Lp220) of the T200 protein complex expressed on a subset of T helper cells. A high affinity (Kₐ approximately 10¹³ to 10¹⁴ M⁻¹) bound conforming of IL 2 receptor (CD25) was purchased from Becton Dickinson (Mountain View, Calif) as a fluorescein conjugate.

Cytotoxicity assay. Peripheral blood mononuclear cells were assessed for NK activity against the CD3-- erythroblastemia cell line K562 using a four-hour ⁵¹Cr-release assay, as previously described. For the sake of clear discussion, we operationally define "NK
activity" in this paper as MHC-nonrestricted cytotoxic activity against K562. Lytic units (LU) were calculated as LU per 10^6 cells for 30% specific lysis of 10^5 target cells.

Effectors. Mononuclear cells from normals and LGL leukemic patients were isolated by Ficoll-Hypaque density centrifugation. In some experiments, mononuclear cells were enriched for LGL by a modification of the technique of Timonen and Saksela. In brief, nylon wool nonadherent mononuclear cells were layered over Percoll step gradients (65%/60%/55%/50%/45%/40% steps) and centrifuged for five minutes at 3,000 g at room temperature. Two-color immunofluorescent flow cytometry analyses of cells from the 50%/55% fraction showed 85% to 95% enrichment in leukemic LGL with a CD3+, HNK-1+ or CD3+, FcR+ phenotype. However, approximately 5% to 15% of cells in this fraction were CD3+, HNK-1- or CD3+, FcR-. Consequently, in some experiments two-color immunofluorescent flow cytometry was utilized to sort for CD3+, HNK-1+ or CD3+, FcR+ leukemic cells.

Induction systems. Effector cells at a concentration of 1 x 10^6/mL were incubated at 37°C in a 95% air/5% CO₂ humidified atmosphere with anti-CD3 MAb, alpha-interferon (IFN; Interferon Sciences, New Brunswick, NJ), or purified IL 2 that had been made lectin-free by chromatographic purification (Lymphocult T-LF, Biotech, Frankfurt, West Germany) for various time periods. Kinetic experiments showed that maximal induction occurred after 48 hours; therefore, subsequent experiments utilized this incubation period. Based on dose-response experiments, a dose of 1 µg/mL of anti-CD3 MAb was used in subsequent experiments. Alpha-IFN was used at a concentration of 1,000 units per 10^6 cells, whereas IL 2 was used at an optimum concentration of 1%, except in one experiment in patient 2, in which an optimum concentration of 5% was used. Other control MAb tested at a concentration of 1 µg/mL included HB10a, 3AC5 anti-Lp220, and 60.3 anti-Lp 95-150. After the incubation period, cells were washed twice, counted, and placed into the NK assay at an appropriate concentration. In addition two-color immunofluorescent flow cytometry analyses were performed after the induction period.

RESULTS

Cell surface phenotype of LGL leukemic cells. One-color analysis showed that LGL leukemic cells from all patients expressed CD2, CD3, CD5, and HNK-1 (Table 1, top). However, when other markers were examined, heterogeneity and unusual surface phenotypes were evident in the leukemic cells: 4 of the 10 patients (1, 3, 8, and 9) had an abnormally high percentage of cells expressing FcR (CD16), while 6 of 10 patients (3, 4, 7, 8, 9, and 10) had increased expression of HLA-DR on leukemic cells. When the leukemic cells were examined by two-color analysis (Table 1, bottom), heterogeneity in surface phenotype was even more evident. For example, many leukemic cells from patients 1, 3, and 8 expressed both CD3 and FcR, a phenotype not generally seen in normal blood lymphocytes (see below). By inference, it was evident that some CD3+, FcR+ leukemic cells from patient 3 also expressed the HLA-DR and HNK-1 markers. Leukemic LGL from patient 5 also had an unusual phenotype, coexpressing CD4 and HNK-1.

A comparison of two-color analyses of normal lymphoid tissues to leukemic cells is shown in Fig 1. Normal peripheral blood mononuclear cells had few CD3+, HNK-1+ cells and no detectable CD3+, FcR+ cells. Normal splenic cells, in contrast, had large numbers of CD3+, HNK-1+ cells; in addition, surface antigen levels of HNK-1 were higher on splenic lymphocytes than on peripheral blood mononuclear cells. The phenotype of LGL leukemic cells resembled that of normal spleen, with large numbers of CD3+, HNK-1+ cells expressing high levels of surface HNK-1. Some leukemias were CD3+, FcR+ (as illustrated by patient 1), a phenotype not readily detectable in normal peripheral blood or splenic mononuclear cells. It should be noted that leukemic cell populations contained some CD3+, HNK-1- or FcR- cells, a point discussed in more detail below.

NK activity of LGL leukemic cells. The expression of FcR and HLA-DR antigens has been associated with NK activity in normal cells, so it was of interest that leukemic LGL from some patients expressed these markers. Previously, we found low NK activity in patients 1, 6, 9, and 10. Table 2 shows that NK activity was also low in other patients, including patients whose cells expressed the FcR and HLA-DR markers. NK activity in patient 7 was also less than normal (data not shown); NK activity in patient 10 was not tested.

Table 1. One- and Two-Color Flow Cytometry Analyses of Peripheral Blood Mononuclear Cells From Patients With LGL Leukemia

<table>
<thead>
<tr>
<th>Antigens</th>
<th>Patients (mean % positive cells)</th>
<th>Normals*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>CD2 (E-receptor)+</td>
<td>59</td>
<td>95</td>
</tr>
<tr>
<td>CD3 (T3)+</td>
<td>81</td>
<td>79</td>
</tr>
<tr>
<td>CD4 (T4)+</td>
<td>12</td>
<td>27</td>
</tr>
<tr>
<td>CD5 (T5)+</td>
<td>41</td>
<td>69</td>
</tr>
<tr>
<td>CD8 (T8)+</td>
<td>37</td>
<td>75</td>
</tr>
<tr>
<td>HNK-1+</td>
<td>65</td>
<td>46</td>
</tr>
<tr>
<td>FcR (CD16)+</td>
<td>73</td>
<td>5</td>
</tr>
<tr>
<td>HLA-DR+</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>CD3+, HNK-1+</td>
<td>56</td>
<td>35</td>
</tr>
<tr>
<td>CD3+, FcR+</td>
<td>64</td>
<td>0</td>
</tr>
<tr>
<td>CD3+, DR+</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>CD8+, HNK-1+</td>
<td>24</td>
<td>41</td>
</tr>
<tr>
<td>FcR+, HNK-1+</td>
<td>ND</td>
<td>1</td>
</tr>
<tr>
<td>FcR+, DR+</td>
<td>ND</td>
<td>0</td>
</tr>
<tr>
<td>CD4+, HNK-1+</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Normal values shown represent mean ± standard deviation in four normal controls.
†ND, not done.
However, we found that LGL leukemic cells could be induced to express NK activity by incubation with anti-CD3 MAb (Table 2). As shown in Fig 2, as little as 50 ng/mL of anti-CD3 MAb produced a dramatic increase in NK activity (uninduced: LU = 0.001; induced: LU = 1.36). The kinetics of augmentation in NK activity are shown in Fig 3. Marked increase in NK activity was observed within 24 hours; maximum induction occurred by 48 hours and subsequently waned. Addition of anti-CD3 MAb at time 0 of a four-hour assay had no effect on NK activity, also indicating that an induction period was necessary to detect optimal activity. Increased NK activity was not accompanied by any obvious increase in cell proliferation as cell numbers remained constant throughout these experiments. A concentration of 1 μg/mL of anti-CD3 MAb and an incubation period of 48 hours were thus selected as optimum conditions for induction. Table 2 shows results of representative experiments utilizing this induction system on peripheral blood mononuclear cells from five LGL leukemia patients. In each experiment there was marked augmentation (9- to >10,000-fold) in NK activity. Of note, this induction was also observed in leukemic cells from patients 2, 4, and 5 which did not express FcR. Similar results were obtained when MOLT-4 cell line was used as target cells (data not shown). Similar experiments showed that anti-CD3 MAb could also increase NK activity in normal peripheral blood mononuclear cells, though this effect was generally much less dramatic (1.3- to 15-fold).

We then examined the effect of other agents on NK induction. As shown in Table 2, NK activity in normal peripheral blood mononuclear cells was also augmented by both IFN and IL 2, as previously reported. IL 2 increased NK activity in some LGL leukemic cells, whereas IFN had no effect. Three other monoclonal antibodies were also examined for their effect on induction (data not shown). MAb recognizing the Lp 95-150 molecular complex (60.3) decreased NK activity in the LGL leukemic cells, as has been described in normal individuals. MAb to Lp220 (3AC5) and HLA-DR (HB10a) had little or no effect on NK activity in leukemic LGL.

Since NK activity in peripheral blood mononuclear cells from LGL leukemia patients could be induced by treatment with either anti-CD3 MAb or IL 2, we examined whether leukemic LGL could be directly activated or whether induction depended on the few remaining normal T cells. Similar to previous results, anti-CD3 MAb induced NK activity in highly purified (85% to 95%) nonadherent low-density CD3+, HNK-1+ leukemic LGL from patient 1 (in two separate experiments, uninduced: LU = 0.01 and 0.01, respectively; induced: LU = 0.33 and 0.91, respectively). Because there was still a small population (5% to 15%) of CD3+ cells with a normal T cell phenotype (ie, HNK-1+, FcR−) remaining in the enriched LGL leukemic populations, it was possible that normal T cells and not the LGL leukemia cells were being induced by anti-CD3 MAb or IL 2. To exclude that possibility, two-color immunofluorescent flow cytometry was utilized to positively sort out CD3+,

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**Table 2: Induction of NK Activity in Peripheral Blood Mononuclear Cells From LGL Leukemia Patients**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Patients</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Media only</td>
<td>3.7</td>
<td>0.01</td>
</tr>
<tr>
<td>Anti-CD3 MAb</td>
<td>33.3</td>
<td>37.0</td>
</tr>
<tr>
<td>IFN</td>
<td>ND†</td>
<td>ND</td>
</tr>
<tr>
<td>IL 2</td>
<td>ND</td>
<td>8.4</td>
</tr>
</tbody>
</table>

*LU/10⁶ cells for 30% specific lysis of 10⁶ target cells. NK activity was assessed after 48 hours incubation with media or various agents, except in patient 2 where incubation period was 60 hours. NK activity of freshly isolated mononuclear cells from patients 1 to 5 was also assessed at time 0; results were similar to those obtained after 48 hours incubation with media alone. Anti-CD3 MAb was used at 1 μg/mL; IFN at 1,000 U/10⁶ cells, and IL 2 at a final concentration of 1% for all patients, except in patient 2 where IL 2 was used at concentration of 5%.†Nylon-wool nonadherent cells.

†ND, not done.
leukemic LGL following incubation with either nonrestricted cytotoxicity against K562 target cells). Augmentation of NK activity by anti-CD3 MAb or IL 2 resulted from a direct effect on CD3+ leukemic LGL and did not require CD3+ cells with a normal T cell phenotype (ie, CD3+, HNK-1−, FcR−); (3) Leukemic LGL and normal lymphocytes differed in their response to alpha IFN; and (4) Despite activation with anti-CD3 MAb or IL 2, leukemic LGL did not express detectable IL 2 receptors.

Since in other lymphoid malignancies, studies on neoplastic cells arrested at certain stages of differentiation have provided insight into the function of their normal counterparts,7–10 we examined the phenotype and function of leukemic LGL. Two-color immunofluorescent flow cytometry analyses revealed heterogeneity of phenotypes expressed on leukemic LGL. However, in all patients the majority of leukemic LGL coexpressed CD3 and HNK-1 and had little NK function in vitro. Confirming previous findings,5,6 we found that cells with this phenotype were present in normal spleen. Most patients with LGL leukemia have splenomegalia, and previous immunopathologic studies have shown involvement of splenic red pulp cords with leukemic CD3+, HNK-1+ cells.24 Taken together, these findings suggest a splenic origin of this disease.

Previous studies had referred to this disease as T gamma lymphoproliferative disorder, since LGL from these patients expressed CD2 and FcR.1 Of note, cells from only four of our patients expressed FcR, thought to be a specific NK marker.21,22 This CD3+, HNK-1+, FcR+ phenotype of some leukemic LGL has not been readily detectable in normal fetal or adult lymphoid tissue,6 although peripheral blood mononuclear cells from 3 of 46 presumably normal individuals were found to have >5% circulating CD3+, FcR+ LGL.27 Because of this phenotypic heterogeneity, it would seem preferable to us to refer to this disease as LGL leukemia rather than T gamma lymphoproliferative disorder.

Leukemic LGL from these patients had little NK activity in vitro, consistent with data showing that normal lymphocytes coexpressing HNK-1 and CD3 (ie, FcR−) have little cytotoxicity.19 However, functional maturation in CD3+ leukemic LGL could be consistently achieved by treatment with anti-CD3 MAb. Anti-CD3 MAb has been reported to induce cytotoxic T lymphocyte activity in normal peripheral blood mononuclear cells.28,29 Furthermore, induction of cytotoxic activity by anti-CD3 MAb in normal LGL has been
response characteristics of this induction system were similar to our findings with leukemic LGL. The dose was used at 1 μg/mL, and IL 2 at a final concentration of 1%.

Purity of these cell populations was >97%.

Lanier et al have induced cytotoxicity by treatment with anti-CD3 MAb used in this study is a potent activator of normal T cell proliferation. Recently it has been shown that certain putative growth factors may instead act as differentiation factors depending on the stage of differentiation of the cell type.

Conversely, normal T cells from these patients (CD3+, HNK-1− cells) could not be similarly activated. Taken together, these findings suggest that anti-CD3 MAb induces cytotoxicity through a direct effect on CD3+, HNK-1+ LGL.

Treatment with IL 2 also induced NK activity in leukemic LGL from 2 of 4 patients. Augmentation of NK activity by IL 2 in normal individuals has been noted previously, though the mechanism has not been clearly ascertained. This effect is not dependent on cell proliferation or on expression of IL 2 receptors that are recognized by the anti-TAC MAb on LGL. Production of gamma IFN after induction with IL 2 has been demonstrated by some investigators; others have not found a contributory role for either alpha or gamma IFN in this induction. CD3−, IL 2 receptor− LGL were shown to respond directly to recombinant IL 2; further work has shown that IL 2−responsive LGL in normal individuals are CD3−, FcR+ (HNK-1+ or −). Of note, no augmentation of NK activity was seen in peripheral blood mononuclear cells coexpressing CD3 and HNK-1 or in CD3+, FcR+ LGL derived from IL 2−dependent short-term cultures.

The mechanism of induction of NK activity in leukemic LGL treated with IL 2 remains to be determined. Similar to findings in normal LGL, we did not find evidence for proliferation (as measured by cell counts) or expression of high-density IL 2 receptors. It is possible that alternative IL 2 receptors not recognized by the anti−IL 2 receptor may be involved in this immunoregulatory effect of IL 2. Alpha IFN does not appear to be involved in this induction system since it did not augment NK activity in leukemic LGL, unlike its effect on normal cells. In contrast to normal peripheral blood LGL, CD3+, HNK-1− leukemic LGL lacking FcR could be directly activated by IL 2. Furthermore, induction of NK activity by IL 2 was also observed in CD3+, FcR+ leukemic LGL.

The activation of normal T cells and CD3+ leukemic LGL differs in that functional maturation of CD3+ leukemic LGL induced by treatment with either anti-CD3 MAb or IL 2 was not accompanied by either cell proliferation or IL 2 receptor induction, even though the anti-CD3 MAb used in this study is a potent activator of normal T cell proliferation. Recently it has been shown that certain putative growth factors may instead act as differentiation factors depending on the stage of differentiation of the cell type.

Table 3. Induction of NK Activity of CD3+ Leukemic LGL Purified by Cell Sorting

<table>
<thead>
<tr>
<th>Cell Population</th>
<th>NK Activity (LU)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Media</td>
</tr>
<tr>
<td>Unsorted</td>
<td>100</td>
</tr>
<tr>
<td>CD3+ Leukemic†</td>
<td>143</td>
</tr>
<tr>
<td>CD3+ Normal‡</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*LU/10^6 cells for 30% specific lysis at 10^6 targets. NK activity was assessed after 48 hours incubation with media or various agents. Anti-CD3 MAb was used at 1 μg/mL, and IL 2 at a final concentration of 1%.

†CD3+ “leukemic” cells were obtained by two-color sorting, and defined as either CD3+, FcR+ (patient 3), or CD3+, HNK-1+ cells (patient 5).

‡CD3+ “normal” cells were CD3+, FcR− cells (patient 3) or CD3+, HNK-1− cells (patient 5) obtained by cell sorting.
induced cell. Our results suggest that whether or not CD3+ cells are induced to proliferate or differentiate by anti-CD3 MAb or IL 2 may depend on their state of differentiation and/or surface phenotype.

The results of these studies indicate that LGL leukemia may serve as a useful model for evaluation of LGL maturation and activation. Furthermore, these induction systems may offer an in vitro method for assessing potential therapy in these patients. The prominent autoimmune manifestations of LGL leukemia suggest an underlying defect in B-cell immunoregulation. Since normal LGL can regulate B cells, the clonal expansion of functionally immature LGL may result in unopposed B cell activity. Indeed, LGL from some of these patients demonstrate impaired suppression of immunoglobulin biosynthesis, in vitro. Augmentation of LGL function by immunomodulatory agents therefore, may be clinically beneficial. Further studies in additional patients with LGL leukemia are needed both to assess fully the in vitro effectiveness of such agents as well as to explain their mechanism of action.

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