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 polyarthritis. 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 the 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+, 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.

A SYNDROME of chronic neutropenia associated with increased numbers of large granular lymphocytes (LGL) has been identified recently. In some of these patients this disease results from a clonal expansion of LGL that is clinically associated with a variety of autoimmune phenomena. Some of these patients form a subgroup of Felty’s syndrome. The CD3(T3)+, HNK-1+ phenotype of the leukemic LGL associated with low natural killer (NK) activity in vitro suggests that these cells may have arisen from normal splenic LGL. In other diseases studies of neoplastic cells “frozen” at stages of maturation have provided important clues concerning the differentiation of their normal counterparts. Therefore, LGL leukemia may provide a model for the analysis of LGL differentiation. In this study, we utilized two-color immunofluorescent flow cytometry analyses together with a variety of in vitro induction systems to examine maturation of leukemic LGL. Our results show that CD3+ leukemic LGL can be directly induced to acquire NK function using either anti-CD3 monoclonal antibody (MAb) or interleukin 2 (IL 2).

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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 10^6 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 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 were performed on cells from the 50%/55% 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 (Lymphocont T-LF, Biotest, 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 (+)</td>
<td>94</td>
<td>97</td>
</tr>
<tr>
<td>CD2 (-)</td>
<td>71</td>
<td>74</td>
</tr>
<tr>
<td>CD3 (+)</td>
<td>68</td>
<td>71</td>
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<tr>
<td>CD3 (-)</td>
<td>59</td>
<td>62</td>
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<tr>
<td>CD4 (+)</td>
<td>84</td>
<td>87</td>
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<td>CD4 (-)</td>
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<td>74</td>
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<tr>
<td>CD5 (+)</td>
<td>86</td>
<td>89</td>
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<tr>
<td>CD5 (-)</td>
<td>67</td>
<td>70</td>
</tr>
<tr>
<td>CD8 (+)</td>
<td>90</td>
<td>92</td>
</tr>
<tr>
<td>CD8 (-)</td>
<td>71</td>
<td>74</td>
</tr>
<tr>
<td>HNK-1 (+)</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>HNK-1 (-)</td>
<td>81</td>
<td>83</td>
</tr>
<tr>
<td>FcR+</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>FcR-</td>
<td>80</td>
<td>82</td>
</tr>
<tr>
<td>HLA-DR+</td>
<td>90</td>
<td>92</td>
</tr>
<tr>
<td>HLA-DR-</td>
<td>71</td>
<td>74</td>
</tr>
</tbody>
</table>

*Normal values shown represent mean ± standard deviation in four normal controls.
nND, not done.
Fig 1. Two-color immunofluorescence flow cytometry analyses of mononuclear cells from normal peripheral blood and spleen, and from LGL leukemia patients utilizing (A) PE-conjugated anti-CD3 MAb (red) versus fluorescein-conjugated HNK-1 MAb (green) and (B) PE-conjugated anti-CD3 MAb (red) versus fluorescein-conjugated anti-FcR MAb (green). 64 x 64 grid plots represent cell numbers on vertical axis versus log of red fluorescence (Y axis) versus log of green fluorescence (X axis), with every 4 to 5 dots representing approximately a doubling of fluorescence.

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 increased cell proliferation as cell numbers remained constant throughout these experiments. A concentration of 1 \( \mu g/mL \) 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 Lp 220 (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+ 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+,

### 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>
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<tbody>
<tr>
<td></td>
<td>1</td>
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</tr>
<tr>
<td>Media only</td>
<td>3.7</td>
<td>0.01</td>
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<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 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 \( \mu g/mL \); 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.
induced in leukemic LGL following incubation with either nonrestricted cytotoxicity against K562 target cells) can be shown). Augmentation of NK activity by anti-CD3 MAb or change in surface antigen expression on induced leukemic density or frequency of expression of either CD3, HNK-1, FcR, or HLA-DR (data not shown). Similarly, there was no change in surface antigen expression on induced leukemic LGL from patient 5 were directly activated by anti-CD3 MAb, no induction was seen in CD3+ T cells not expressing FcR or HNK-1 from these patients (Table 3). In patient 3, CD3+, FcR+ leukemic LGL were also directly activated by IL 2; again there was no effect of IL 2 on normal FcR- T cells from this patient.

We then examined whether induction in NK activity was associated with a change in surface antigen phenotype of the leukemic LGL. Using directly FITC-conjugated MAb and flow cytometric analysis, we found that induction with anti-CD3 MAb in leukemic LGL from patient 1 with a CD3+, HNK-1+ phenotype did not change the density or frequency of expression of either CD3, HNK-1, FcR, or HLA-DR (data not shown). Similarly, there was no change in surface antigen expression on induced leukemic LGL from patient 5 (CD3+, HNK-1-, FcR-) in particular, there was no induction of FcR on these cells (data not shown). Augmentation of NK activity by anti-CD3 MAb or IL 2 was also not associated with expression of high-density IL 2 receptors on HNK-1+ leukemic cells (Fig 4).

**DISCUSSION**

The results of this study demonstrate several new findings in LGL leukemia. (1) NK activity (defined as MHC-nonrestricted cytotoxicity against K562 target cells) can be induced in leukemic LGL following incubation with either anti-CD3 MAb or IL 2; (2) Activation with either 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,17-19 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,8,6 we found that cells with this phenotype were present in normal spleen. Most patients with LGL leukemia have splenomegaly, and previous immunopathologic studies have shown involvement of splenic red pulp cords with leukemic CD3+, HNK-1+ cells.7,4 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 pg/mL, and IL 2 at a final concentration of 1%

Purity of these cell populations was >97%.

CD3+, HNK-1+ leukemic LGL that are either FcR+ or FcR- can be findings, in addition, demonstrate that CD3+, HNK-l + 2-dependent cultures derived from normal individuals.' Our anti-CD3 MAb, in CD3+, FcR+ cells from short-term IL effect seen at higher concentrations of MAb. Recently with as little as 10 ng/mL of anti-CD3 and with a plateau seen in normal LGL, with augmentation occurring not noted, although this effect depended on normal T cells, in contrast to our findings with leukemic LGL. The dose-response characteristics of this induction system were similar to that seen in normal LGL, with augmentation occurring with as little as 10 ng/mL of anti-CD3 and with a plateau effect seen at higher concentrations of MAb. Recently Lanier et al have induced cytototoxicity by treatment with anti-CD3 MAb in CD3+, FcR+ cells from short-term IL-2-dependent cultures derived from normal individuals.27 Our findings, in addition, demonstrate that CD3+, HNK-1+ leukemic LGL that are either FcR+ or FcR- can be directly activated by treatment with anti-CD3 MAb. However, normal T cells from these patients (CD3+, HNK-1- or CD3+, FcR-) 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.30,31 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.32 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.

Fig 4. Lack of expression of IL 2 receptor on leukemic LGL from patient 1 even after activation with anti-CD3 MAb. One- (A and B) and two-color (C and D) flow cytometry analyses of uninduced (A and C) and induced (B and D) leukemic LGL utilized PE-conjugated anti-IL 2 receptor MAb (red) and fluorescein-conjugated HNK-1 MAb (green). 64 by 64 grid plots represent cell numbers on vertical axis versus log of red fluorescence (Y axis) versus log of green fluorescence (X axis) with every 4 to 5 dots representing approximately a doubling of fluorescence. LU/10⁶ effector cells for 30% killing of 10⁶ target cells.

![Flow cytometry analysis](image)

<table>
<thead>
<tr>
<th>Cell Population</th>
<th>Media</th>
<th>Anti-CD3</th>
<th>IL 2</th>
<th>Patient 3</th>
<th>Media</th>
<th>Anti-CD3</th>
<th>IL 2</th>
<th>Patient 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unsorted</td>
<td>100</td>
<td>151</td>
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<td>0.77</td>
<td>41.7</td>
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<tr>
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<td>250</td>
<td>294</td>
<td>&lt;0.001</td>
<td>0.008</td>
<td>0.001</td>
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<tr>
<td>CD3+ Normal‡</td>
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<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td></td>
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</table>

*LU/10⁶ cells for 30% specific lysis at 10⁶ 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).
‡Purity of these cell populations was >97% in both cases.
§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,


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Induction of NK activity in large granular lymphocyte leukemia: activation with anti-CD3 monoclonal antibody and interleukin 2

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