Activation Signals Leading to Proliferation of Normal and Leukemic CD3⁺ Large Granular Lymphocytes

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The activation signals leading to proliferation of normal and leukemic CD3⁺ large granular lymphocytes (LGL) were studied in vitro. Anti-CD3 monoclonal antibody (MoAb) alone (P < .01) and recombinant interleukin-2 (IL-2) alone (P < .01) caused significant stimulation of peripheral blood mononuclear cells (PBMC) from four CD3⁺ LGL leukemia patients, as measured in a ³H-thymidine incorporation assay. Recombinant interleukin-4 (IL-4) alone had no effect (P = .11). The combination signals of anti-CD3 MoAb and either IL-2 or IL-4 produced a proliferative response greater than anti-CD3 MoAb alone (P < .01) or lymphokine alone (P < .01). Leukemic LGL, purified by two-color sorting, were subsequently activated by anti-CD3 MoAb and IL-2 and assessed for DNA content by viable Hoechst No. 33342 (HO) staining. Results of these studies demonstrated that leukemic LGL were stimulated directly by anti-CD3 MoAb and IL-2, with the percentage of cells in cell cycle (S + G2/M) ranging from 16% to 72%. Normal CD3⁺ LGL were also stimulated to enter the cell cycle by anti-CD3 and IL-2. These results show that leukemic LGL proliferate in vitro after activation through the T-cell receptor and/or lymphokines.

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MATERIALS AND METHODS

Patients. All patients met clinical criteria for diagnosis of LGL leukemia with LGL counts ranging from 4,335 to 9,470/µL (compared with normal: 223 ± 99 cells/µL, n = 10). In each patient, at least 80% of the circulating peripheral blood mononuclear cells (PBMC) were LGL. Previous publications have detailed the lymphocyte surface markers of PBMC from patients 1 through 3. All three patients studied had evidence for clonal rearrangement of T-cell receptor β gene.

Lymphocyte phenotyping. PBMC were first isolated from whole blood using Ficoll-Hypaque density gradient centrifugation. These cells were subsequently analyzed for the presence of cell surface antigens using an EPIC cytofluorometer (Couler, Hialeah, FL) and a panel of directly conjugated and unconjugated MoAb. MoAb (1 µg/mL), recombinant IL-2 at 500 IU/mL (Amgen, Thousand Oaks, CA) and recombinant IL-4 at 200 U/mL (kindly provided by Dr Craig Smith, Immunex, Seattle, WA) were used in cell cultures to induce proliferation. The affinity purified G19-4 anti-CD3 MoAb used in cell cultures to induce proliferation was kindly provided by Ed Clark, University of Washington, Seattle. List mode data was collected and analyzed using Reproman software, gating on viable cells. Results were reported as percentage positive based on the threshold values for fluorescence determined from suitable isotype-specific control antibodies.

Proliferative assay. PBMC were adjusted to 1.0 × 10⁶ cells per milliliter in RPMI containing 10% fetal calf serum (FCS) (GIBCO, Grand Island, NY). One hundred microliters of these cells was transferred to the microtiter wells of a 96-well microtiter plate (Costar, Cambridge, MA). To these wells were added anti-CD3 MoAb (1 µg/mL), recombinant IL-2 at 500 IU/mL (Amgen, Thousand Oaks, CA) and recombinant IL-4 at 200 U/mL (kindly provided by Dr Craig Smith, Immunex, Seattle, WA) either alone or in combination. All stimuli were tested in triplicate. The microtiter plates were subsequently incubated at 37°C and 5% CO₂/air humidified atmosphere for 72 hours. The wells were then pulsed with ³H-thymidine for 18 hours, and subsequently harvested with a PhD cell harvester and counted.

Purification of lymphoid subsets. Highly purified lymphoid subpopulations were obtained by sorting using the following procedure. Patient cells, 10 × 10⁶ were washed in 2× sterile phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA)
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(Sigma, St Louis, MO) and centrifuged at 1,000 rpm for 10 minutes. The resulting pellet was resuspended in 200 µL of PE-conjugated Leu1 MoAb (anti-CD5) (Becton-Dickinson), incubated for 30 minutes at 4°C, washed one time with PBS/BSA, and resuspended in 200 µL of FITC-Leu7 for an additional 30 minutes at 4°C. Anti-CD5 MoAb was used for cell sorting rather than anti-CD3 MoAb since we wished to avoid activating cells during the sort. CD5+ cells from these patients overlap with CD3+ cells (not shown). These conjugated MoAbs were first dialyzed exhaustively against PBS/BSA buffer to remove sodium azide and subsequently filter-sterilized. Dual labeled cells were washed one time and finally resuspended at 3.0 x 10⁶ cells per milliliter and were ready for sorting. Sorting was accomplished using an EPIC cytofluorometer (Coulter). Windows were set to collect two nonoverlapping populations: CD5+ CD57- (T cells) or CD5+ CD57+ (LGL) cells. These purified lymphoid subsets (95% enriched as determined by reanalysis) were subsequently cultured at a concentration of 1.0 x 10⁶ cells per milliliter in RPMI medium containing 10% FCS with or without anti-CD3 MoAb (1 µg/mL) and IL-2 (500 U/mL) in 25 cm² flasks (Corning, Corning, NY). After 3 days of culture at 37°C, the cells were prepared for cell cycle analysis. PBMC derived from normal individuals were first cultured for 3 days and then sorted as described above.

Cell cycle analysis. Cells were washed one time with 1× PBS containing 2% FCS, and resuspended in 200 µL of Hoechst No. 35342 (HO) (Sigma) at 10 µg/mL. These cells were incubated at 37°C for 40 minutes in 5% CO₂/air humidified atmosphere, washed one time in 1× PBS containing 2% FCS and 10 µg of HO per milliliter, resuspended in 500 µL of this same buffer, and immediately analyzed on an EPIC cytofluorometer using a UV laser (360 nm). Listmode data was collected and then analyzed using ReproMan software. Results are reported as percent of cells in resting phase (G₀/G₁), stationary phase (S), G₂/M, or total cells in cycle (S + G₂/M).

Statistics. To test whether anti-CD3 MoAb or lymphokine stimulated proliferation, a stratified Wilcoxon Rank Sum statistic was used to compare experimental results with those values obtained in the medium control. These statistical analyses were also used to compare results obtained using combinations of activation signals with those obtained using the activation signal alone.

RESULTS

Lymphocyte phenotype. All four patients had CD3+ LGL leukemia with greater than 87% of PBMC reacting with anti-CD3 MoAb. These cells were TCRαβ and TCRγδ.

Analysis of proliferative signals. Results are summarized in Fig 1. Anti-CD3 MoAb alone or IL-2 alone caused significant proliferation of PBMC from the LGL leukemia patients. In contrast, IL-4 alone did not act as a proliferative signal. IL-2 plus IL-4 produced a somewhat increased proliferative response, when compared with IL-2 alone, but this was not statistically different from the effect produced by IL-2 alone (P = .99). The combination of anti-CD3 MoAb with either IL-2 or IL-4 produced synergistic effects in increasing the proliferative response when compared with the effect produced by any of these stimuli used alone. Anti-CD3 MoAb plus IL-2 and IL-4 produced the maximum proliferative response. Our finding of increase proliferation after activation by anti-CD3 MoAb contrasts to that of our previous study in which we examined the induction of cytotoxicity produced by anti-CD3 MoAb. In that study, we found no increase in cell numbers up to 96 hours after activation of leukemic LGL by anti-CD3 MoAb. In the current study, we used thymidine incorporation and cell cycle analyses to assess increased cell synthesis at 72 hours after activation. The different methodologies might explain the discordant results. It is conceivable that enhanced cell death is occurring simultaneously with increased cell synthesis, resulting in no net increase in cell number. Alternatively, the period of observation may have been too short to detect an increase in cell numbers.

Cell cycle analyses of purified CD5+ CD57+ (T cells) and CD5+CD57+ (LGL) populations from patient and normal PBMC. We previously observed that leukemic LGL are directly induced by anti-CD3 MoAb to acquire cytotoxic function. Therefore, it was likely that the leukemic LGL were also being activated to proliferative by these signals. However, it was possible that the observed proliferative response was attributable to the small number of cells with the normal T cell phenotype (ie, CD3+CD57+). To address this issue, we examined the proliferative effects of anti-CD3 MoAb plus IL-2 on highly purified lymphoid subsets from these patients. Cells from three patients were first sorted into CD5+CD57+ and CD5+CD57+ populations. Analysis of these sorted populations showed them to be greater than 95% pure. These cells were subsequently cultured with anti-CD3 MoAb and IL-2 for 3 days at 37°C and 5% CO₂, air
humidified atmosphere, treated with HO, and analyzed for cells in cycle. A representative histogram showing DNA content obtained from cytofluorometric analysis is shown in Fig 2 and the results are summarized in Table 1. In each patient, leukemic LGL (CD5⁺CD57⁻) were directly stimulated to enter the cell cycle, although the percentage of cells in S + G/M varied among patients. The finding in patient 2 that a high percentage of both sorted populations entered into cell cycle when compared with unsorted cells activated in the same manner is difficult to explain. However, this finding is not due to the sorting process itself causing activation into the cell cycle, since in all cases 100% of sorted cells in medium alone remained in G0/G1. Our inability to detect DNA synthesis in unactivated leukemic LGL in peripheral blood (Fig 2) agrees with the findings of a previous study. In that study, however, a high proportion of leukemic splenic LGL were actively in cell cycle, suggesting that the spleen may be either a site of activation in vivo or a site to which activated leukemic LGL home.

Cells with a normal T-cell phenotype in these patients (CD5⁺CD57⁺) also directly entered the cell cycle in varying percentages. The activation signal produced by anti-CD3 MoAb and IL-2 may be explained, in part, by our previous observation that anti-CD3 MoAb upregulates the number of p75 IL-2 receptors on leukemic LGL, since the proliferative effects of IL-2 are mediated through this receptor. Whether anti-CD3 MoAb has a similar effect on IL-4 receptors remains to be studied.

The activation signals for normal CD3⁺ LGL are not well understood, since these cells constitute only 2% of normal PBMC. CD3⁺ LGL mediate non-MHC-restricted cytotoxicity, which can be activated by anti-CD3 MoAb. Our studies showed that normal CD3⁺ LGL are activated to enter the cell cycle by the combined signals of anti-CD3 MoAb and IL-2. These results suggest that antigen binding to TCR (as mimicked by anti-CD3 MoAb in vitro) accompanied by lymphokine secretion might be a mechanism for activation for both normal and leukemic CD3⁺ LGL. The unique subset of normal CD3⁺ LGL may represent a population of in vivo primed cytotoxic T lymphocytes, with antigen specificity directed against viral targets. Although there is no direct evidence to explain the leukemic state, preliminary data indicate that some patients are infected with an HTLV-1-like retrovirus. Lymphokine stimulation has been proposed as a mechanism of HTLV-1 induced acute T cell leukemia (ATL) as evidenced by (1) upregulation of p55 IL-2 receptor subunits and autocrine secretion of IL-2 by ATL cells, and (2) proliferation of ATL cells in response to IL-2. We speculate that LGL leukemia might result from an expansion of LGL responding to foreign antigen and lymphokine secretion.

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