Cytokine Influence on Killing of Fresh Chronic Lymphocytic Leukemia Cells by Human Leukocytes

By Dževad Ćemelíc, Barbara Daday, Tin Han, and Louis Vaickus

The feasibility of combining the Lym-1 monoclonal antibody (MoAb) with interferon-γ (IFN-γ) in the treatment of chronic lymphocytic leukemia (CLL) was evaluated. We used an in vitro tumor lysis model that incorporated fresh CLL cells from 21 different patients as targets for two distinct normal human leukocyte effector subsets, neutrophils, and peripheral blood mononuclear cells (PBMCs). Lym-1 antigen (Lym-1-Ag) expression varied greatly and did not correlate with the expression of other CLL-associated antigens such as CD6, CD19, or HLA-DR. CLL cells were not lysed by neutrophils alone or with IFN-γ in the absence of Lym-1. Neutrophil Lym-1–dependent cytotoxicity (ADCC) in the absence of IFN-γ was weak and inconsistent. IFN-γ exposure induced MoAb-dependent lysis of 80% of 21 CLL targets and resulted in an eightfold augmentation of neutrophil ADCC against the remainder. Cytotoxicity correlated directly and positively with Lym-1-Ag expression. Confirmation of the need for interaction between neutrophil IgG Fc receptors (FcRγs) and the Fc portion of the Lym-1 MoAb was obtained by demonstrating that purified Staphylococcus aureus Protein A (SpA) inhibited ADCC. IFN-γ exposure caused no consistent alterations in Lym-1-Ag expression on CLL cells so that target antigen upregulation was unlikely to account for augmentation of neutrophil ADCC. PBMCs alone, exposed to interleukin-2 (IL-2) or IFN-γ, or with Lym-1 in the presence or absence of IL-2 or IFN-γ were unable to lyse CLL targets. PBMCs were able to kill Raji Burkitt lymphoma cells in conjunction with Lym-1, so their ability to interact with Lym-1–coated targets and their lytic functions appeared intact. These results emphasize the importance of examining fresh tumor cells with different leukocyte effector subsets before designing a clinical trial that combines a therapeutic MoAb with a cytokine.

THERAPY WITH monoclonal antibodies (MoAbs) has resulted in limited responses in virtually all reported trials.1 In an effort to improve these response rates, investigators have attempted to enhance the potency of the antibody itself (by linking it to lethal substances such as radioisotopes, drugs, or toxins) or to improve the host’s ability to eliminate antibody-coated tumor targets by augmenting cellular immunity with recombinant cytokines. Three preliminary clinical trials have failed to demonstrate a significant advantage to cotreatment with interferon-α (IFN-α) and anti-idiotypic MoAbs in B-cell lymphomas,2 IFN-γ, and the CO17 1A MoAb in patients with colon cancer,3 and IFN-α and an anti-G3 MoAb in melanoma.4 These trials were based on preclinical data that suggested the efficacy of cytokine plus MoAb cotherapy either in vivo in a mouse model or in vitro using human effector cells and tumor cell lines.

In an effort to choose the optimal cytokine for a clinical trial, we have examined several for their ability to enhance cell-mediated killing of human tumor cell lines. Previously, we characterized the influence of interleukin-2 (IL-2) on peripheral blood mononuclear cell (PBMC)-mediated killing.5 We discovered that PBMCs exposed to IL-2 either in vivo or in vitro displayed markedly enhanced MoAb-independent and MoAb-dependent (ADCC) lysis of tumor cell lines, including those derived from chronic lymphocytic leukemias (CLL).

In separate studies, neutrophils were consistently potent mediators of ADCC but only in the presence of IFN-γ.6,7 PBMCs treated with IFN-γ produced significantly greater ADCC than untreated PBMC, but the degree of augmentation was far less than that observed with similarly treated neutrophils. Enhancement of ADCC by these cytokines (IL-2 and PBMCs; IFN-γ and neutrophils) shared many common properties that would be desirable in a clinical trial: brief cytokine exposure primed the effector cells, clinically attainable concentrations were effective, and ADCC was augmented at low antibody concentrations and low effector-to-target ratios.

In this study, we compared PBMC and neutrophil killing of fresh CLL targets in ADCC and antibody-independent cytotoxicity. Neutrophils were able to mediate killing of fresh CLL cells only in the presence of MoAb. The addition of IFN-γ markedly augmented neutrophil ADCC. In contrast, IL-2– and IFN-γ–stimulated PBMCs were virtually devoid of killing activity. The implications of these findings for therapy of cancer with MoAb/cytokine combinations are discussed.

MATERIALS AND METHODS

Human cell line and culture conditions. The Raji human Burkitt lymphoma cell line was obtained from the American Type Culture Collection (Rockville, MD). All cells were cultured in RPMI 1640 medium, 8% heat-inactivated (56°C for 45 minutes) fetal calf serum (FCS), L-glutamine (2 mmol/L), nonessential amino acids (0.2 mmol/L), and HEPES buffer (0.02 mol/L) without antibiotics (GIBCO, Grand Island, NY), hereafter referred to as complete medium (CM). Cells were incubated in a humidified air environment containing 7.5% CO2 at 37°C. Cell viability was determined by trypan blue exclusion and was usually greater than 95%.

MoAbs and other reagents. Lym-1 is a murine IgG2a MoAb originally described by Epstein et al8 and was provided by D. Shochat (Lederle Laboratory Division of American Cyanamid).
Corporation, Pearl River, NY). Lym-1 was greater than 90% pure antibody as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Lym-1 reacts with a variant class II (HLA-DR family) molecule present on many B-cell malignancies including non-Hodgkin’s lymphoma (NHL) and acute and chronic leukemias. Lym-1 reacts weakly with a limited number of normal cells (B cells, cells of monocyte/macrophage origin), the Lym-1 antigen (Lym-1-Ag) is not shed from the surface of cells, and Lym-1 has been used to treat patients with B-cell malignancies in its unconjugated and $^{111}$I-conjugated forms. MoAbs reactive with CD5 (Leu 1), CD3 (Leu 4), CD19 (Leu 12), CD14 (Leu M3), CD20 (Leu 16), and HLA-DR were purchased from Becton Dickinson (Mountain View, CA); anti-α, anti-λ antibodies and fluoresceinated F(ab)2 goat antimouse IgG were purchased from TAGO, Inc (Burlington, CA). Lym-1 was sterilized with 0.2-μm filters and stored at −20°C. Staphylococcus aureus Protein A (SpA; Sigma, St Louis, MO) binds to the Fc region of Lym-1 and was used to inhibit ADCC by blocking Fc-FeR interactions. SpA was mixed with Lym-1 for at least 10 minutes before addition to ADCC assays.

**CLL patients.** After informed consent, 21 patients with CLL, 11 females and 10 males, were entered into this study. The diagnosis of CLL was established by standard clinical and laboratory criteria by one of us (T.H. or L.V.) and by immunophenotypic analyses using a panel of B- and T-cell markers (Table 1 and Fig 1). The percent of Lym-1-positive cells was determined for each patient proximate to the assay.

Fresh CLL cells were obtained from whole blood by venipuncture and collected into sterile Vacutainer tubes (Becton Dickinson) containing preservative-free heparin. Blood was diluted with RPMI 1640 medium 1:1 and layered on Ficoll-Hypaque, specific gravity 1.077 (Pharmacia, Piscataway, NJ). CLL cells were obtained from theuffy coat as described. Viability was always greater than 98%.

**Effector cells.** Effector cells were obtained from the whole blood of normal donors after informed consent, as described. Briefly, whole blood was layered onto neutrophil isolation medium (Los Alamos Diagnostics, Los Alamos, NM) and effector cell subsets isolated by centrifugation at 400g for 30 minutes at room temperature (RT). The upper buffy coat consisted of PBMCs and the lower of neutrophils. The purity of these preparations was as follows (mean percent ± SD): PBMCs were 86.4 ± 3.0% lymphocytes, 9.3% ± 1.9% monocytes, and 4.3% ± 1.9% neutrophils. Neutrophils were 98.6% ± 0.9% neutrophils, 0.2% ± 0.2% monocytes, and 2.0% ± 1.1% lymphocytes (n = 10). These results were obtained from an ES/T 8000 Analyzer (Ortho Diagnostics, Raritan, NJ) and confirmed by microscopic inspection of Wright’s stained cytopsin preparations and immunofluorescence on a flow cytometer (FACScan; Becton Dickinson, Sunnyvale, CA) as described.

**Recombinant human cytokines.** IFN-γ was purchased from Collaborative Research Inc (Bedford, MA). Activity was determined by inhibition of encephalomyocarditis (EMC) virus propagation in WISH amnion cells. The IFN-γ was greater than 98% pure by SDS-PAGE, had 2.9 × 10^5 U/mg protein, 0.069 endotoxin units/5 × 10^5 U, and its amino acid composition correlated greater than 99% with native human IFN-γ. Recombinant human IL-2 was provided by Hoffman-La Roche (Nutley, NJ), had a specific activity of 1.5 × 10^5 U/mg protein, undetectable endotoxin levels, and was greater than 95% pure.

**Flow cytometric analysis.** Surface marker phenotyping of cells was performed by indirect immunofluorescence on a flow cytometer as described. A minimum of 2,500 cells were counted per test. Immunofluorescence was expressed as percent positive cells and by

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**Summary**

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Immunophenotyping was performed proximate to the time of cytotoxicity assays. Values represent percent Lym-1-Ag or surface marker (eg, CD5) positive cells out of the total number of CLL cells. The second value represents mean fluorescence of the positive cells. A dash means not done.
Fig 1. Flow cytometric analysis of surface markers estimated by indirect immunofluorescence of patient ZG CLL cells. Results are expressed as percent positive cells and mean log fluorescence (each figure, upper right hand corner). The ordinate represents relative cell number. For each marker, 4,000 cells were analyzed relative to a negative antibody control adjusted so that ≤ 5% of cells were positive.

**RESULTS**

Immunophenotyping of freshly isolated CLL cells was performed on the same day as cytotoxicity assays (Table 1). Epstein et al had previously reported that Lym-1 stained 40% of CLLs (n = 10). We needed to determine the level of Lym-1-Ag expression (as estimated by MF) and the percent of total CLL cells that were positive. Immunophenotyping would also determine whether the majority of the targets were tumor cells. The Lym-1-Ag was detected in a proportion of every CLL sample that we surveyed, but the percent of positive cells varied widely (mean 41%; range, 13% to 76%). The intensity of expression was less variable with a MF average of 421 and a range of 425 to 640. Lymphocyte and natural killer cell markers such as CD5 (MF average 461), CD19 (MF average 652), and HLA-DR (MF average 652), but was not detected on as many CLL cells (CD5, CD19, and HLA-DR average 421, 20, and 51, respectively). Of interest, Lym-1 binds to a variant HLA-DR molecule and it appears to be discordantly regulated compared with nonpolymorphic DR antigens. Most of the cells that were used as targets in these experiments were lytic activity. A minimum of four E:T ratios were used to calculate LU; however, in most experiments five or more E:T ratios were used. The viability of neutrophils at the end of the ADCC assay was between 95% and 100%, and they were metabolically active (as determined by reduction of the tetrazolium salt MTT, as described). In addition, the cytoplasmic contents of neutrophils disrupted by sonication did not cause 51Cr release; thus, CLL cell killing was specific and not due to release of intracellular contents from dead or dying effector cells. This finding is similar to what we reported for the effect of neutrophil contents (obtained by freeze-thaw) on Raji targets. Statistical analyses. Statistical analyses were accomplished by use of the paired t-test and the Pearson correlation coefficient.
tumor cells because their phenotype was consistent with CLL cells (CD5+, CD19+, HLA-DR+) and they exhibited light chain excess. A representative, composite phenotype is shown in Fig 1. Contamination with the major lymphocyte subset in normal PB, T cells, was minor (mean of 11% of the total were CD3+) and monocyte content (as estimated by CD14 expression) was negligible (Fig 1).

Neutrophil-mediated killing of CLL cells was examined (Fig 2 and Table 2). Neutrophils alone or with IFN-γ were unable to lyse CLL cells. Neutrophils plus Lym-1 in the absence of IFN-γ lysed CLL targets only when Lym-1-Ag expression was high (patients RG and ZG). The addition of IFN-γ consistently induced or upregulated (fourfold to 17-fold increase) Lym-1–dependent neutrophil-mediated tumor lysis. The varying levels of cytotoxicity that were observed suggested that there was a correlation with CLL Lym-1-Ag expression. Indeed, when LU or percent cytotoxicity at a 100:1 E:T ratio was compared with Lym-1-Ag expression there was a strong positive correlation (Fig 3A, P < .001, r = .677 for cytotoxicity; Fig 3B, P < .001, r = .653 for LU). The data in Fig 3A and B are a compilation of the results presented in Table 2 and, thus, effector cell variability has not been completely eliminated. However, effectors from one donor (LV) were used against five different targets and there was a strong correlation between lysis and Lym-1 antigen expression. Examined in another way, there was a 329% increase in lysis when CLL targets with greater than 50% Lym-1-Ag expression were compared with those with less than 50%. Also, CLL targets with less than 50% Lym-1-Ag expression all had LU values of zero when incubated with neutrophils and Lym-1 in the absence of IFN-γ.

The tumoricidal capability of PBMCs was examined simultaneously (Fig 4). PBMCs alone, with IL-2, or with IFN-γ were unable to lyse CLL cells in an antibody-independent fashion. PBMC ADCC was negligible with Lym-1 alone or in combination with IFN-γ (P not significant compared with PBMCs alone). Cytotoxicity by PBMCs plus IL-2 and Lym-1 was slightly better, but the majority of this apparent increase was due to assays that used ZG CLL cells that expressed the greatest amount of Lym-1-Ag. For most CLL targets, the combination of PBMCs plus IL-2 and Lym-1 was ineffective. Indeed, if this one patient’s cells are excluded from the analysis, cytotoxicity was negligible for the PBMC plus Lym-1 and IL-2 combination (LU values were [mean ± 1 SD] 0.7 ± 2.3 and percent cytotoxicity at 100:1 E:T ratio values were 13 ± 11). The same PBMCs were simultaneously tested for their ability to lyse Raji targets. PBMCs were always able to mediate ADCC against Raji cells with Lym-1 (not shown) or in conjunction with Lym-1 plus IL-2 (Fig 4), thus, their lytic functions were intact.

Cytotoxicity was examined after 4 and 16 hours to determine whether duration of contact between effectors and CLL targets influenced lysis (Table 3). As in our prior studies, a longer duration of contact between effectors and targets resulted in a higher level of lysis against high or low expressors of the Lym-1-Ag. As before, neutrophils were much more effective mediators of CLL lysis than PBMCs. To confirm that IFN-γ–enhanced, neutrophil-mediated lysis of CLL targets was due to interactions between

### Table 2. IFN-γ Augments Neutrophil-Mediated Killing of CLL Cells

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<th>CLL Cells Donors</th>
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*a Values show percent of CLL cells that expressed Lym-1-Ag (n = 21 from 11 CLL donors).

†Results are expressed as LU 33%/10^7 effector cells. In all experiments, neutrophils (NEUTs) alone, IFN-γ alone, or Lym-1 plus IFN-γ in the absence of neutrophils were ineffective. A dash means not done.

‡Only percent cytotoxicity at 100:1 E:T ratio is shown because low effector cell yield did not allow a complete LU 33%/10^7 cells to be performed.
NEUTROPHIL KILLING OF FRESH CLL TARGETS

Fig 3. Killing of CLL cells by IFN-γ-treated neutrophils correlated positively with Lym-1-Ag expression. Each dot represents one experiment (n = 23 for A and n = 21 for B). The ordinate in A represents cytotoxicity at a 100:1 E:T ratio and in B represents LU 33/10' effectors. Twenty-one of 23 values in A are from the experiments summarized in Table 2, while all 21 values in B are from Table 2. The abscissae show percent Lym-1-Ag-positive cells out of total CLL cell pool as determined by fluorescence on a flow cytometer (2,500 cells counted per experiment and compared with a negative antibody control).

DISCUSSION

The major findings of this study are the following: (1) neutrophils were proficient killers of CLL cells via ADCC in the presence of IFN-γ; (2) the degree of CLL target susceptibility to neutrophil-ADCC was highly dependent on Lym-1-Ag expression; (3) CLL cells were resistant to PBMC-mediated ADCC in the presence or absence of IFN-γ or IL-2; and (4) fresh CLL cells were not susceptible to lysis by neutrophils or PBMCs in the presence of IFN-γ or IL-2 via MoAb-independent mechanisms.

The inability of unconjugated MoAbs to consistently

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The inability of unconjugated MoAbs to consistently

Fig 4. IL-2 and IFN-γ effect on PBMC-mediated CLL cell killing. The ordinates show cytotoxicity at a 100:1 E:T ratio (upper panel) and LU 33/10' effector cells (lower panel). The abscissae lists the various reagents added to PBMCs and target cells. The values represent the mean of 17 experiments ± 1 SD.
produce responses in clinical trials may be due to any of the following: high levels of circulating free antigen, formation of human antimouse antibodies (HAMA), antigenic modulation, poor tissue penetration, insufficient level of tumor cell target antigen expression, or emergence of antigen-negative mutants. However, a relative or absolute failure of the contention that host effector mechanisms must be activated for optimal antitumor response. Therefore, a strategy that uses recombinant cytokines to augment or sustain a host cellular immune response in conjunction with an antitumor MoAb would seem to be a reasonable option for improving response rates in the serotherapy of cancer.

Many investigators have shown that a plethora of cytokines (IFN-α, IFN-γ, IL-2, tumor necrosis factor, granulocyte-macrophage colony-stimulating factor [GM-CSF], macrophage-CSF [M-CSF]) can augment ADCC. However, most of these studies are not applicable to anticancer serotherapy because one or more of the following was an integral component of the model: nonhuman targets; red blood cell (RBC) targets; xenogeneic antisera; or broadly reactive MoAbs (eg, anti-class I). We and others have examined in vitro systems comprised of human effector cells, tumor cell lines, and recombinant cytokines in conjunction with therapeutic MoAbs in an attempt to use this preclinical information to rationally design clinical trials.

We had previously demonstrated that PBMCs exposed to IL-2 in vitro or PBMCs harvested from cancer patients receiving IL-2 in vivo displayed a marked enhancement of ADCC and antibody-independent lysis of a panel of Lym-1-Ag expressing human tumor cell lines, including three response33,34 results of clinical trials with the CAMPATH rat MoAbs and rat-human chimeras also lent support to the contention that host effector mechanisms must be activated for optimal antitumor responses. Therefore, a strategy that uses recombinant cytokines to augment or sustain a host cellular immune response in conjunction with an antitumor MoAb would seem to be a reasonable option for improving response rates in the serotherapy of cancer.

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different CLL cell lines. IFN-γ had a similar effect on PBMC ADCC. However, under identical treatment conditions PBMCs were unable to lyse fresh CLL cells (Fig 4). A similar dichotomy between the susceptibility of cultured cell lines and fresh tumors to lysis has been observed with IL-2-activated natural killer (NK) cells and leukemic targets. The reasons for this are unknown and the possibilities include the following: (1) Although IFN-γ enhanced neutrophil ADCC against CLL cells, IFN-γ and IL-2 may have decreased PBMC lytic functions. This possibility is unlikely because we and others have reported that IL-2 and IFN-γ have consistently and significantly augmented PBMC ADCC against a variety of human tumor targets, including, as mentioned above, CLL cell lines. IFN-γ and IL-2 have been reported to exert a protective effect on fresh tumor cells and tumor cell lines and render them resistant to NK or lymphokine-activated killer (LAK) cell-mediated antibody-independent lysis. Lahat et al reported that IL-2 and IFN-γ enhanced NK activity against fresh hairy cell leukemia targets, but that exposure of targets to these cytokines before the assay decreased their susceptibility to lysis. IFN-γ was reported to have a similar effect on melanoma cell lines in PBMC ADCC, especially at IFN-γ concentrations greater than 100 U/mL. Unless this protective effect is somehow unique to a PBMC/CLL combination, it cannot explain our results because IFN-γ did not have this effect on any other effector/target combination (Table 3). (3) CLL cells are susceptible to neutrophil lytic mechanisms but resistant to those mediated by PBMCs. While this hypothesis is possible, because neutrophil and PBMC lytic mechanisms are different, we feel that a more likely explanation is that (4) PBMC lytic functions were not triggered by the Lym-1/Lym-1-Ag array on the surface of most CLL cells. We feel that this possibility is likely because PBMCs were capable of ADCC against CLL cells, albeit minimal, and there was evidence of enhancement by IL-2 (Fig 4). However, conditions had to be optimal (presence of IL-2, highest Lym-1-Ag-expressing CLL targets, 100:1 E:T ratio) for PBMC ADCC.

The reasons why neutrophil lytic mechanisms were more efficient at killing CLL targets are not clear. We have demonstrated that the effector cell within the PBMC leukocyte subfraction exclusively used the type III IgG Fc receptor (FcγR), CD16, to mediate lysis of Raji targets in conjunction with Lym-1. The Lym-1/Lym-1-Ag surface complex on fresh CLL cells may not sufficiently perturb PBMC CD16 such that a stimulus threshold is exceeded with subsequent triggering of the lytic mechanism. In contrast, we have demonstrated a specific upregulation of CD64 (FcγRI) by IFN-γ that appears to be critical for neutrophil ADCC with Lym-1. Thus, triggering of the neutrophil’s lytic mechanism via CD64 may be more efficient at killing fresh CLL cells. Although this study did not address which leukocyte FcγRs were functionally important in lysis of CLL cells, it is reasonable to conclude that they were the same. Further examination of antibody-dependent leukocyte killing of CLL cells will be necessary to identify functionally important FcγRs.

Neutrophils may be an ideal effector cell to exploit in vivo against CLL in combination with a MoAb for a variety of reasons: (1) neutrophils have not been reported to be impaired in CLL patients, as opposed to descriptions of numerical and functional deficiencies of T and NK cells; (2) they are potent mediators of antibody-dependent lysis of fresh CLL cells in conjunction with IFN-γ (Table 2); (3) they are the most abundant leukocyte subset in humans and can be quantitatively augmented by growth factors such as G-CSF and GM-CSF; (4) neutrophils have been shown to be capable of infiltration into tumor sites in experimental nonhuman animals and in humans; and (5) CLL patients are much less likely to form HAMA than other cancer patients, and this may allow multiple treatment courses.

IFN-γ has advantages and disadvantages as a cytokine to combine with an MoAb for CLL therapy. IFN-γ monotherapy has resulted in responses in CLL. Levels of IFN-γ used in our study can be attained in vivo. Schiller et al reported that IFN-γ levels of approximately 170 to 670 U/mL were detected 120 hours after initiation of a 5-day continuous intravenous infusion at 700 μg/d/patient. It is difficult to assess whether this level would be reached with IFN-γ monotherapy because IFN-γ was coadministered with IFN-β in this study. Importantly, IFN-γ side effects are treatable, generally tolerable, and reversible with few reports of grade III toxicity.

Dose-dependent neutropenia is the main disadvantage of IFN-γ if neutrophil ADCC augmentation is the goal. However, neutropenia may not preclude combination with a MoAb because the optimal antiproliferative dose of IFN-γ may be higher than the concentration required to enhance neutrophil ADCC, ie, the optimal immunomodulatory dose. Results consistent with this contention have been reported for monocye ADCC.

In summary, we have shown the complexities involved in designing clinical protocols based on preclinical data involving MoAbs and recombinant cytokines. It seems clear that if an in vitro model is to have utility in the design of clinical trials, or have predictive value in the follow-up of alterations in immune parameters after cotreatment of patients with MoAbs and cytokines, the model should incorporate components that are as relevant as possible. This will allow for the optimal design of treatment strategies that exploit particular immune effector mechanisms such as neutrophil ADCC. The results of this study suggest that IFN-γ would be a reasonable cytokine to combine with Lym-1 in a phase I immunotherapy trial of selected CLL patients.

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Cytokine influence on killing of fresh chronic lymphocytic leukemia cells by human leukocytes

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