RAPID COMMUNICATION

Interleukin-4 Induces Programmed Cell Death (Apoptosis) in Cases of High-Risk Acute Lymphoblastic Leukemia

By Atsushi Manabe, Elaine Coustan-Smith, Masa-aki Kumagai, Frederick G. Behm, Susana C. Raimondi, Ching-Hon Pui, and Dario Campana

We investigated the effects of interleukin-4 (IL-4) on the survival of leukemic and normal B-cell progenitors cultured on bone marrow stroma. IL-4 (at 100 U/mL) was cytotoxic in 16 of 21 cases of B-lineage acute lymphoblastic leukemia, causing reductions in CD19+ cell numbers that ranged from 50% to greater than 99% (median 83.5%) of those in parallel cultures not exposed to the cytokine. All nine cases with the t(9;22)(q34;q11) or the t(4;11)(q21;q23), chromosomal features that are often associated with multidrug resistance and a fatal outcome, were susceptible to IL-4 toxicity. IL-4 cytotoxicity resulted from induction of programmed cell death (apoptosis); there was no evidence of cell killing mediated by T, natural killer, or stromal cells. IL-4 cytotoxicity extended to a proportion of normal B-cell progenitors. After 7 days of culture with IL-4 at 100 U/mL, fewer CD19+, CD34+ normal lymphoblasts (the most immature subset) survived: in five experiments the mean (±SEM) reduction in cell recoveries caused by IL-4 was 60.0% ± 6.0%. By contrast, reductions in recovery of more differentiated bone marrow B cells (CD19+, CD34+, surface Ig+) were low (6.6% ± 2.2%; \( P < 0.001 \) by t-test). Our findings indicate that IL-4 is cytotoxic for human B-cell precursors and support clinical testing of IL-4 in cases of high-risk lymphoblastic leukemia resistant to conventional therapy.

© 1994 by The American Society of Hematology.
V tissue culture medium. The normal and leukemic immature B cells were then resuspended in AIM-V and 1 to 10^6 cells were placed on the stromal layer in each well. In cultures of normal marrow B cells, CD19 expression was consistently greater than 90%, whereas in leukemic cultures percentages of CD19 positivity were below 90%. Recombinant human IL-4 (Genzyme, Cambridge, MA; Cat. No. 2181-01; specific activity = 10^6 U/mg) was then added to the cultures at various concentrations, as indicated in Results. Cultures were incubated for 7 days at 37°C, 5% CO_2, and 90% humidity. IL-4 activity was neutralized with a goat antihuman IL-4 antiserum (R & D System, Minneapolis, MN; Cat. No. AB-204-NA). In some experiments, parallel cultures of B-progenitor cells were treated with dexamethasone (Sigma, St Louis, MO; Cat. No. D4902) at concentrations ranging from 0.001 to 10μg/mL.

Conditioned medium was prepared by incubating confluent stromal layers and normal peripheral blood lymphocytes with IL-4 (100 U/mL) for 7 days at 37°C. The culture supernatant was collected, passed through a 0.22-μm filter, and frozen at −70°C. The supernatant was thawed immediately before use, and 25-μL aliquots were added to B-lineage ALL cells on stromal feeder layers.

Flow cytometry and DNA fragmentation assay. Cell numbers and phenotypes were determined by flow cytometry when cultures were established and again after 7 days, as described. Briefly, stromal cultures were transferred to Falcon tubes (Becton Dickinson, Lincoln Park, NJ; Cat. No. 2052). B-lineage ALL cells were incubated with CD19 (Leu 12) monoclonal antibody (MoAb) conjugated to fluorescein isothiocyanate (FITC) and CD3 (Leu 4) antibody conjugated to phycoerythrin; normal CD19^+ BM cells were incubated with a combination of CD19 conjugated to peridinin chlorophyl protein, CD34 antibody conjugated to phycoerythrin, and a mixture of goat antihuman Ig light-chain antisera conjugated to FITC. All MoAbs and isotype-matched unreactive controls were purchased from Becton Dickinson (San José, CA); goat antisera were from Southern Biotechnology Associates (Birmingham, AL). After two washes in PBS with 0.2% bovine serum albumin and 0.2% sodium azide, the cells were resuspended in 0.5% paraformaldehyde and analyzed with a FACScan flow cytometer and Lysis II software (Becton Dickinson). In each case, we imposed "gates" around the areas of the light-scatter dot plot that comprised the vast majority of leukemic cells at the beginning of the cultures and used them to enumerate cells with the predetermined light-scattering properties that were present after 7 days of culture with or without IL-4.

The results were corrected for the percentage of cells in each sample expressing different antigens. The formula (no. of cells recovered with IL-4)/ (no. of cells recovered without IL-4) was used to calculate relative cell recovery after IL-4 treatment. All results are reported as the mean of duplicate experiments (intraassay variability <10%).

Flow cytometry and DNA fragmentation assay. Cell numbers and phenotypes were determined by flow cytometry when cultures were established and again after 7 days, as described. Briefly, stromal cultures were transferred to Falcon tubes (Becton Dickinson, Lincoln Park, NJ; Cat. No. 2052). B-lineage ALL cells were incubated with CD19 (Leu 12) monoclonal antibody (MoAb) conjugated to fluorescein isothiocyanate (FITC) and CD3 (Leu 4) antibody conjugated to phycoerythrin; normal CD19^+ BM cells were incubated with a combination of CD19 conjugated to peridinin chlorophyl protein, CD34 antibody conjugated to phycoerythrin, and a mixture of goat antihuman Ig light-chain antisera conjugated to FITC. All MoAbs and isotype-matched unreactive controls were purchased from Becton Dickinson (San José, CA); goat antisera were from Southern Biotechnology Associates (Birmingham, AL). After two washes in PBS with 0.2% bovine serum albumin and 0.2% sodium azide, the cells were resuspended in 0.5% paraformaldehyde and analyzed with a FACScan flow cytometer and Lysis II software (Becton Dickinson). In each case, we imposed "gates" around the areas of the light-scatter dot plot that comprised the vast majority of leukemic cells at the beginning of the cultures and used them to enumerate cells with the predetermined light-scattering properties that were present after 7 days of culture with or without IL-4.

The results were corrected for the percentage of cells in each sample expressing different antigens. The formula (no. of cells recovered with IL-4)/ (no. of cells recovered without IL-4) was used to calculate relative cell recovery after IL-4 treatment. All results are reported as the mean of duplicate experiments (intraassay variability <10%).

For cell-cycle analysis, mononuclear cells were stained with CD19 antibody conjugated to FITC, fixed, and permeabilized as described by Schmid et al. After treatment with DNase-free RNase (11.25 Kunitz units; Boeringer Mannheim, Indianapolis, IN), the cells were
IL-4 CYTOTOXICITY TO LEUKEMIC CELLS

Fig 1. Dose dependency of IL-4-induced cytotoxicity in stromal layer cultures of leukemic lymphoblasts. Numbers correspond to cases listed in Table 1.

labeled with propidium iodide (10 µg/mL; Calbiochem, La Jolla, CA). Cells were analyzed with a FACSscan instrument and CellFit software (Becton Dickinson).

DNA fragmentation was investigated as previously described.11,12

RESULTS

Cytotoxicity of IL-4 to leukemic B lymphoblasts. After 7 days of culture on allogeneic BM stromal layers, the number of leukemic lymphoblasts per case of B-lineage ALL ranged from 19% to 199% (median, 74%) of that originally plated. In 16 of the 21 cases, IL-4 treatment (100 U/mL) reduced the recovery of CD19+ cells by 50% to greater than 99% (median, 83.5%) compared with results for parallel cultures not exposed to the cytokine (Table 1). The cytotoxic effect of IL-4 was dose dependent (Fig 1) and was completely abrogated by addition of a neutralizing antibody (not shown).

Cases sensitive to IL-4 were heterogeneous with respect to immunophenotype, percentage of cells in S phase, and age of the patients at diagnosis. Notably, however, all six cases with the t(9;22)(q34;q11), or “Philadelphia chromosome,” and the three cases with t(4;11)(q21;q23) abnormalities were susceptible to IL-4–induced cytotoxicity. Comparison of the cytotoxicity of IL-4 with that of dexamethasone in the same culture system was performed in two cases. These experiments indicated a higher cell kill by the cytokine, even when the steroid was added at the highest concentration, 10 µmol/L (data not shown). The remaining five cases, as well as the four experimental cell lines (380, Nalm 6, 697, and REH), were completely resistant to the toxic effects of IL-4.

Cytotoxicity of IL-4 is caused by induction of apoptosis. The low spontaneous proliferative activity of ALL blasts (mean percentage of cells in S phase = 3.7%, Table 1)13 made it unlikely that the large decreases in cell numbers seen after IL-4 treatment were caused solely by growth inhibition. Rather, the IL-4–induced changes in the light-scattering properties of leukemic cells, as detected in all cases by flow cytometry, resembled those induced by antileukemic drugs that act by triggering apoptosis: eg, corticosteroids, teniposide, and anti-FAS antibody16-18 (and A.M., D.C., unpublished observations, December 1992). These irreversible effects consisted of decreased forward light scatter, indicative of a reduction in cell size, accompanied by increased orthogonal light scatter, indicative of augmented intracellular granularity (Fig 2). In cell sorting experiments, populations that displayed similar light-scattering properties had fragmented DNA, the hallmark of apoptosis19,20 (and A.M., D.C., unpublished observations, February 1992). Changes in cell morphology observed in all cases in situ with an inverted microscope (ie, nuclear fragmentation in cells with an apparently intact cell surface membrane) and in three cases after Wright-Giemsa staining of cytospins (ie, nuclear fragmentation and numerous apoptotic bodies in stromal macrophages) were also typical of apoptosis.20 Evidence of massive DNA fragmentation was detected after 48 hours of treatment with IL-4 and persisted for 7 days, accompanied by a progressive decrease of viable cells in three cases studied (Fig 3). Finally, in three cases studied, treatment with IL-4 appeared to block

Fig 2. Flow cytometric analysis of IL-4 effects on ALL cells. Dot plots depict forward light scatter (FSC = cell size; X axis) and side light scatter (SSC = cell granularity; Y axis). Decreasing proportions of lymphoblasts retain the light scattering properties of viable cells with increasing concentrations of IL-4. Cultures were performed for 7 days on stromal layers as described in Materials and Methods.
the entry of cells into S phase of the cell cycle and was accompanied by an increase in cells with subnormal DNA content (Fig 4), consistent with cell death in G0/G1.

Cytotoxicity of IL-4 in culture is not mediated by T, natural killer (NK), or stromal cells. We also considered the possibility that induction of apoptosis in B-lineage ALL cases was mediated by IL-4–stimulated T lymphocytes and NK cells present, albeit in very small numbers (ie, <1%), in the culture. Thus, in two cases (nos. 1 and 6; Table 1) we re-investigated IL-4 cytotoxicity on purified CD19+ cells, among which less than 0.1% CD3+ or CD56+ lymphocytes were detected. Percent cell killing by IL-4 (100 U/mL) in these tests was identical to that listed in Table 1. Moreover, we tested the effects of conditioned medium prepared from normal peripheral blood lymphocytes cultured with 100 U/mL of IL-4 for 7 days. Control supernatant from cultures without IL-4 did not affect ALL blast viability in the two cases studied, whereas in cultures with the IL-4–conditioned medium, the relative recoveries of CD19+ cells were 91% and 89% (Fig 5). The minor cytotoxic effect of the conditioned medium was completely eliminated by preincubation with a neutralizing antibody to IL-4 (Fig 5).

Identical results were obtained with conditioned medium from IL-4–treated stroma. Cytotoxicity produced by this medium (42% and 50% relative cell recoveries in the two cases studied) was abrogated by neutralizing antibody to IL-4 (Fig 5). To investigate whether factors bound to the stromal layers but not secreted in the supernatant could be responsible for apoptotic cell death of ALL blasts, we treated stromal layers with IL-4 for 4 days and then added leukemic blasts with or without neutralizing antibody to IL-4. Results showed that cytotoxicity was completely abrogated by the antibody (Fig 5).

Finally, a stroma-independent cell clone derived from one of the cases studied (no. 1, Table 1) was susceptible to IL-4 in cultures performed in the absence or in the presence of stromal cells, as shown by flow cytometric and morphologic changes typical of apoptosis, and cell reductions in the presence of IL-4 under both culture conditions (percent cell killing = 75% with 50 U/mL and 85% with 100 U/mL). Taken together, these results indicate that IL-4 induces apoptosis of B-lineage ALL blasts directly rather than by stimulating accessory cells.

Cytotoxicity of IL-4 to normal BM B cells. We used triple-color immunofluorescence and flow cytometry to quantify the effects of IL-4 on the recovery of cells at different stages of normal B-cell differentiation after culture. Cells were stained with antibodies to CD19, CD34, and surface Ig (sIg) simultaneously, permitting recognition of relatively immature (CD34+) and mature (sIg+) subpopulations, as well as a third group of cells (CD19+, CD34+, and sIg-) most likely at an intermediate level of differentiation. In all samples, there were no cells labeled by both CD34 and surface Ig. After 7 days of culture with or without IL-4 at 100 U/mL, fewer CD19+, CD34+ lymphoblasts (the most immature subset) survived in the IL-4–treated cultures; mean (±SEM) reduction in cell recoveries in the five experiments was 60.0% ± 6.0% (Table 2). By contrast, the reduction in the number of mature surface Ig+B lymphocytes recovered in the same cultures was only 6.6% ± 2.2% (P < .001 by t-test; Table 2). Reduction in recovery of BM B cells at an intermediate stage of differentiation, lacking CD34 and surface Ig, was variable (38.0% ± 11.0%) and tended to be higher than that seen in more mature sIg+B lymphocytes (P < .03). In all samples investigated, the intensity of expression of surface Ig was increased approximately twofold by IL-4 treatment, as previously reported.21

DISCUSSION

Our findings show that IL-4 directly induces apoptosis in the blast cells from a substantial fraction of cases of B-lineage ALL, the most common form of leukemia in children. Because the cytokine was also toxic to normal B-cell
progenitors but not for mature B lymphocytes, this activity must be selective for differentiation stage. While earlier studies failed to detect any effect of IL-4 on DNA synthesis and on the colony-forming ability of normal and leukemic human B lymphoblasts, recent reports have emphasized the suppressive effect of IL-4 on spontaneous proliferation of both normal and leukemic B-cell progenitors. In our study, the induction of apoptosis by IL-4 was accompanied by a block in cell-cycle progression before the cells entered S phase, indicating that the decrease in proliferation observed by other investigators could be largely caused by rapid induction of programmed cell death.

Corticosteroids and several other antineoplastic drugs kill leukemic cells by inducing programmed cell death. Here we show by stringent criteria that IL-4 also induces apoptosis in B-lineage ALL cells, a strikingly different effect than that seen in B-cell chronic lymphocytic leukemia, where IL-4 protects sIg+ lymphocytes from apoptosis. The molecular basis for this opposite effect needs to be elucidated, but Fanslow et al postulated that binding of IL-4 to low-affinity receptors, abundant on immature B cells, may result in signals different than those occurring when the cytokine interacts with both low- and high-affinity receptors expressed by mature B cells.

Because susceptibility to IL-4–induced cell death decreases during normal differentiation, it may be that sensitivity to IL-4 is higher in ALL cases originating from more undifferentiated progenitor cells. However, we could not find obvious correlations between sensitivity to IL-4 and expression of leucocyte differentiation antigens on leukemic blasts. In contrast to findings by Pandrau et al, who reported IL-4–induced inhibition of leukemic cell proliferation only in cases lacking CD20 expression, more than half of the sensitive cases in our series had greater than 20% CD20+ lymphoblasts. It is tempting to speculate that amounts and affinity of surface receptors for IL-4 correlate with sensitivity to the toxic effects of the cytokine, and that the complete lack of IL-4–induced cytotoxicity in 5 of the 21 cases studied was simply caused by a lack of functional IL-4 receptors. This possibility is attractive and requires further investigation; however, surface expression of IL-4 receptors does not necessarily correspond to susceptibility to IL-4, as illustrated by studies of receptor-positive cell lines.

Whether the effects of IL-4 on immature B cells cultured on BM stroma are direct or mediated by unknown factors...
secreted by the stromal cells themselves, as suggested by Billips et al.\(^2\) in experiments with the murine stroma line S17, remains controversial. Our experiments using stroma-conditioned medium and IL-4-treated stroma failed to show an anti-immature B-cell activity other than IL-4 in the supernatants, indicating that no other factors with anti-B-cell activity were secreted and that the observed effects were exclusively caused by the cytokine. In addition, the cytotoxic effects of IL-4 were also observed in the ALL cell clone growing in vitro without stromal cells.

The effects of IL-4 on normal B-cell progenitors seeded onto stromal layers were specific for differentiation stage. The most immature identifiable B-lineage cells in BM, those expressing CD34, were clearly susceptible to the suppressive effects of IL-4. We cannot state with certainty whether these were caused by inhibition of proliferation or induction of apoptosis, although results with phenotypically identical leukemic cells would suggest that IL-4 may be cytotoxic to immature normal B-cell progenitors. We think it unlikely that induction of differentiation could have caused the decrease of CD19\(^+\), CD34\(^+\) precursors because a corresponding increase of CD19\(^+\), CD34\(^-\), slg\(^-\) cells was not observed. In addition, Punnonen et al.\(^9\) have recently shown that IL-4 may induce B-cell precursors to differentiate into Ig-secreting cells only after they have reached the CD34\(^-\) pre-B stage. Studies with murine long-term lymphoid cultures have indicated that IL-4 may promote the development of undifferentiated, steroid-sensitive, lymphoblasts which lack B220 and have incomplete (DJ) IgH gene rearrangements.\(^9\) In our study, we observed that a proportion of normal CD34\(^+\), CD19\(^+\), slg\(^-\) cells were spared by IL-4, at concentrations that could eliminate greater than 90% of leukemic blasts with an equivalent phenotype. We speculate that some of the CD34\(^+\), CD19\(^+\), slg\(^-\) cells in our cultures, apparently insensitive to the cytotoxic effects of IL-4, may represent the most undifferentiated cells equivalent to B220\(^-\) murine B-cell progenitors.

To our knowledge, this study provides the first experimental evidence of a cytokine that triggers programmed cell death in leukemic lymphoblasts from patients, a finding that has clear clinical implications. Currently, about 30% of children and 65% of adults with ALL develop recurrences of their disease.\(^30,31\) Patients with the Philadelphia-chromosome or the 4;11 translocation, representing approximately 6% of children and 30% of adults with ALL, have a dismal prognosis; most of them succumb to leukemia despite intensive treatment.\(^32\) We think it noteworthy that most of our cases, including the nine with these adverse cytogenetic features, were susceptible to IL-4-induced apoptosis. In phase I clinical trials of IL-4 in patients with solid tumors, cytotoxic serum levels of the cytokine were achieved at tolerable administered doses.\(^33\) Thus, biologic chemotherapy of ALL with IL-4 is feasible and our findings provide experimental support for clinical trials of IL-4 in patients with high-risk ALL.

**ACKNOWLEDGMENT**

We thank J. Gilbert for critical review and editorial suggestions.

**REFERENCES**


IL-4 CYTOTOXICITY TO LEUKEMIC CELLS


Interleukin-4 induces programmed cell death (apoptosis) in cases of high-risk acute lymphoblastic leukemia

A Manabe, E Coustan-Smith, M Kumagai, FG Behm, SC Raimondi, CH Pui and D Campana