Interleukin-4 Enhances the Survival of Severe Combined Immunodeficient Mice Engrafted With Human B-Cell Precursor Leukemia

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Human interleukin-4 (hIL-4) has been shown to inhibit the growth in vitro of cells from patients with acute lymphoblastic leukemia (ALL). With the aim of determining whether this cytokine might be useful in the treatment of patients with ALL, the effects of hIL-4 on human B-cell precursor ALL engrafted in severe combined immunodeficient (SCID) mice were examined. The inhibition of [3H] thymidine uptake of primary ALL cells by hIL-4 was maintained following engraftment and passage of leukemia in SCID mice. Five of seven xenograft leukemias showed significant inhibition in vitro by hIL-4 at concentrations as low as 0.5 ng/mL; furthermore, hIL-4 counteracted the proliferative effects of IL-7. When used to treat two human leukemias engrafted in SCID mice, hIL-4 200 μg/kg/d, as a continuous 14-day subcutaneous infusion, suppressed the appearance of circulating lymphoblasts and extended survival of mice by 39% and 108%, respectively, the first demonstration of IL-4 activity against human leukemia in vivo. The mean steady-state hIL-4 level in mouse plasma during the infusion was 1.46 ng/mL (SEM ± 0.14 ng/mL), which was similar to concentrations found to be effective in vitro. ALL cells obtained from mice relapsing after hIL-4 treatment continued to show inhibition by the cytokine in vitro. These data suggest that IL-4 may be useful in the treatment of patients with ALL.

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MATERIALS AND METHODS

Engraftment of human leukemia in SCID mice. Bone marrow (BM) or peripheral blood (PB) was collected from seven patients with B-cell precursor ALL, including one patient with ALL arising as blast crisis of chronic myeloid leukemia (CML) (Table 1). Mononuclear cells were separated by ficoll-metrizoate density gradient centrifugation (Lymphoprep; Nycomed AS, Oslo, Norway) and washed in RPMI 1640 medium (GIBCO [BRL], Paisley, UK). Cells were washed in RPMI 1640 medium (GIBCO [BRL], Paisley, UK). Cell concentration was adjusted to 1 x 10^8/ml for injection into SCID mice.

SCID mice were obtained from the National Institute of Medical Research (Mill Hill, UK) and maintained in individually ventilated cages. Female mice received 2 Gy irradiation (60Co source, 60 cGy/min) and were injected intravenously (IV) the same day with 1 to 3 x 10^7 primary human BM or PB leukemia cells. Animals later showed infiltration of BM by human ALL lymphoblasts and gross splenic enlargement with infiltration by greater than 95% human cells. Immunophenotype and karyotype analysis of xenograft cells was unchanged from the primary material. Subsequently, 2 x 10^7 human ALL xenograft cells obtained from mouse spleen were passage to further irradiated SCID mice.

Preparation of leukemia samples for in vitro testing. Single-cell suspensions of human ALL xenograft cells were obtained by passing mouse spleen through a 180-μm wire mesh filter. Cells were washed twice in RPMI 1640 medium.

Where the sensitivity of primary ALL leukemia cells to cytokines...
was investigated in vitro, cryopreserved lymphoblasts collected by leukopheresis were thawed, washed, and mononuclear cells separated by density gradient centrifugation. Mononuclear cells were depleted of monocytes by plastic adherence and of T cells by rosette formation using sheep red blood cells (SRBCs). Cells were cultured in plastic tissue culture flasks (Nunc, Roskilde, Denmark) at a density of 2 to 5 x 10^6 cells per mL in 40 mL RPMI medium with 10% fetal calf serum (FCS, GIBCO). After incubation at 37°C for 2 hours in 5% O_2, 10% CO_2, 85% N_2, 0.3 mL of packed SRBCs (Serotec, Oxford, UK) was added and incubation continued for a further 10 minutes. Cell suspensions were centrifuged at 250g for 10 minutes then held at 4°C for 90 minutes. The cell pellet was resuspended in medium and mononuclear cells separated from T-cell rosettes by density gradient centrifugation.

In vitro effects of IL-4, IL-7, and the combination of IL-4 with IL-7. Human ALL xenografts obtained from mouse spleen (first through fifth passage), with greater than 95% viability assessed by trypan blue exclusion, were incubated in 96-well plates (Falcon 3072; Becton Dickinson, Lincoln Park, NJ) at a concentration of 1 to 2 x 10^5/mL in RPMI 1640 medium with 10% FCS. huIL-4 (specific activity 10 U/ng; Schering-Plough, Bury St Edmonds, UK) was added to give a final concentration range of 0.1 to 50 ng/mL, and with and without 200 U/mL human IL-7 (Immunex, Seattle, WA). Six 200-μL wells were used per group. Plates were incubated at 37°C in 5% O_2, 10% CO_2, 85% N_2. Primary human leukemia cells (ALLl) were likewise incubated with huIL-4 0.1 to 50 ng/mL.

After 3 days' incubation, 1 μCi [3H] thymidine (Amersham, Little Chalfont, UK) was added per well and plates incubated for a further 8 hours. Cells were harvested onto glass-fiber filters using an automatic cell harvester (Inotech, Wohlen, Switzerland) and radioactive uptake measured directly from the filters using the Inotech Automatic Filter Counting System.

Treatment of human leukemia in SCID mice using IL-4. Female SCID mice were irradiated and injected via tail vein with 2 x 10^7 fresh human ALL xenograft cells from mouse spleen. Fourteen days later, osmotic pumps (Alzet model 2002; Charles River, Margate, UK) were implanted subcutaneously (sc) to produce a steady 14-day infusion. Pumps were weighed to ensure complete filling. For each experiment, six mice received huIL-4 200 μg/kg/d in phosphate-buffered saline (PBS) with 0.1% bovine serum albumin (Sigma, Poole, UK), and six control mice were implanted with pumps delivering diluent only. After completion of the infusion, pumps were removed and examined for complete delivery of their contents.

Flow cytometry analysis of human leukemia cells in SCID mouse blood. At 7- to 10-day intervals, 20 μL of blood was obtained from mice and collected into 30 μL PBS with 50 U/mL preservative-free heparin. Fluorescein-conjugated antibody to human class I HLA (W6/32; Sera-lab, Crawley Down, UK) was added at a concentration of 1:20 for 30 minutes on ice. Two milliliters of lysis solution (0.037 g disodium EDTA, 1.0 g potassium bicarbonate, 8.3 g ammonium chloride in 1 L) was then added for 10 minutes. After washing, samples were resuspended for flow cytometry analysis using an Ortho Cytoron Absolute flow cytometer (Ortho Diagnostic Systems, Raritan, NJ). The proportion of fluorescein-labeled cells was determined within a large gate encompassing the nucleated mouse and human cell populations. Normal SCID mouse blood was included as a negative control with all analyses.

Measurement of huIL-4 levels in SCID mice. At day 21, 1 week after commencing huIL-4 treatment, blood samples were obtained from mice for measurement of huIL-4 levels. Samples were immediately centrifuged and plasma removed for storage at −80°C. A human immunoassay kit (Quantikine D4000; R&D Systems, Oxon, UK) and manufacturer's methodology was used to measure huIL-4 levels. The range of the assay was 0.0312 ng/mL to 2 ng/mL.

**RESULTS**

Inhibition of ALL cell growth in vitro by IL-4. Using cells obtained at presentation from the patient, the sensitivity of ALLl to incubation with huIL-4 was investigated. At concentrations of 1 ng/mL and above, greater than 85% inhibition of [3H] thymidine uptake was observed (Fig 1).

SCID mice were engrafted with leukemia from 7 patients with B-cell precursor ALL (Table 1). Single-cell suspensions were prepared from mouse spleen and the effects of in vitro exposure to huIL-4 were studied.

In total, of the seven human ALL xenografts tested, five showed significant inhibition of DNA synthesis by huIL-4.
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1 ng/mL, with [3H] thymidine uptake reduced to between 18% and 88% of control (ALL1-4 and 7; P < .01 analysis of variance [ANOVA]; Fig 2). Inhibition was also significant with huIL-4 at 0.5 ng/mL but not 0.1 ng/mL (data not shown). As these xenograft cells proliferated poorly in vitro, the lymphoproliferative cytokine IL-7 was added to produce a stimulus for leukemia proliferation and to assess the effects of huIL-4 under such conditions. IL-7 has proliferative effects in vitro on leukemia cells from most patients with ALL.20,21

When incubated with human IL-7 200 U/mL used alone, DNA synthesis was increased 1.5- to 3.5-fold compared with controls for 5 of the 7 cases (ALL1-3, 5, and 6; Fig 2).

For all cases, when huIL-4 1 ng/mL was added to IL-7 200 U/mL, thymidine uptake was reduced to between 9% and 74% of uptake of IL-7-alone cultures (P < .01). In particular, the addition of huIL-4 counteracted the marked stimulatory effects of IL-7 on the two leukemias (ALL1 and 2), which were later treated with huIL-4 in SCID mice. Although huIL-4 alone did not significantly inhibit thymidine uptake of the two Philadelphia chromosome-positive (Ph) ALL cases (ALL5 and 6), nevertheless the cytokine did counteract the proliferative effects of IL-7 (Fig 2).

Comparison of the in vitro effects of IL-4 on primary and passaged human leukemia. It was necessary to establish that the sensitivity of ALL cells to growth inhibition by huIL-4 was not altered by passage within SCID mice. The effects of huIL-4 and IL-7 were compared for primary leukemia cells (ALL1) obtained from the patient and ALL1 xenograft samples from the 5th and 22nd passage in SCID mice (Fig 3). IL-4 produced similar levels of DNA synthesis inhibition in the three samples, both used alone and in combination with IL-7. The stimulatory effects of IL-7 were also retained after multiple passage of the leukemia.

Effects of IL-4 on human ALL engrafted into SCID mice. The in vivo effects of huIL-4 were tested for two human leukemias engrafted in SCID mice which had been shown to be inhibited by huIL-4 in vitro. These were an early B-cell precursor ALL established from a 36-year-old man (ALL1, sixth passage) and a pre-B ALL established from a 2-year-old female (ALL2, fourth passage).

Fig 2. The effects in vitro of IL-4 (■, 1 ng/mL), IL-7 (□), 200 U/mL, and the combination of IL-4 with IL-7 (■) on human xenograft leukemia. [3H] Thymidine uptake assay results after 3 days culture are expressed as percentage of control culture cpm ± SEM.

Fig 3. The effect of passage in SCID mice on IL-4 inhibition and IL-7 stimulation of human leukemia ALL1. [3H] Thymidine uptake assay results are expressed as percentage of control culture cpm ± SEM. (□), Control; (■), IL-4 1 ng/mL; ( ), IL-7 200 U/mL; ( ), IL-4 + IL-7.
Fig 4. Treatment of human ALL1 leukemia in SCID mice using human IL-4 (six mice per group). Percentage of human cells in mouse blood ± SEM (A) and animal survival (B).

0.96 to 1.98 ng/mL for the six mice tested (Fig 6). Human IL-4 was not detectable in PB of control mice. To investigate IL-4 levels with bolus dosing, six SCID mice were given daily sc injections of huIL-4 200 μg/kg for 1 week and PB samples were taken immediately before and 1 and 4 hours after dosing. The predose level was 0.003 ng/mL, below the range of the assay. One hour after dosing, plasma huIL-4 was 17.2 ng/mL (SEM ± 3.2 ng/mL), but at 4 hours had decreased to 0.14 ng/mL (SEM ± 0.06 ng/mL).

The in vitro sensitivity of ALL1 and ALL2 to IL-4 is retained in IL-4–treated animals. For both ALL1 and ALL2, human ALL xenograft cells were obtained from spleens of animals that had relapsed after huIL-4 treatment and the sensitivity of these cells to huIL-4 and IL-7 was compared with ALL1 and ALL2 cells from untreated animals.

Both ALL1 and ALL2 xenograft cells from huIL-4–treated animals continued to show significant inhibition of DNA synthesis by huIL-4 1.0 ng/mL both alone (P < .05) and in combination with IL-7 (P < .01). However, the degree of inhibition was reduced compared with that observed using cells obtained from untreated mice (Fig 7). IL-4 inhibition of thymidine uptake by untreated ALL1 xenograft cells was 82% compared with 40% inhibition of treated ALL1 xenograft cells (Fig 7A). For ALL2 xenograft cells, huIL-4 inhibition was 51% for previously untreated cells and 19% for treated cells (Fig 7B).

DISCUSSION

We have shown that huIL-4 suppressed human B-cell precursor ALL engrafted in SCID mice and improved animal survival. This finding correlated with the observed effects of IL-4 on ALL cells in vitro.

Primary ALL1 cells were inhibited in vitro by huIL-4 0.5 ng/mL and the inhibitory effects were maintained after engraftment and multiple passage of this leukemia in SCID mice. Significant inhibitory effects of huIL-4 were also seen for 5 of 7 human xenograft leukemias established from patients with B-cell precursor ALL, at concentrations of 0.5 ng/mL and above. Similar concentrations of huIL-4 have been reported by Manabe et al11 to produce apoptosis of B-precursor ALL cells when cultured on BM stroma. Under suspension culture conditions, as used in the current study, ALL cells survive poorly. It is possible that in suspension culture a high background rate of apoptosis of leukemic cells masked the effects of IL-4 in some cases.

Clearly there are aspects of both the human and the SCID mouse environment that support proliferation of human ALL. These factors are ill-defined. In humans it is possible that the lymphoproliferative cytokine IL-7 is involved in ALL cell growth in vivo. Therefore, we studied the effects of costimulation of ALL cells with human IL-7 and huIL-4. The inhibitory effects of huIL-4 continued to be observed in leukemia cells stimulated by IL-7. In two cases IL-4 counteracted the increased DNA synthesis seen with IL-7, whereas IL-4 used alone had shown no effect.

The efficacy of huIL-4 treatment of ALL in vivo was tested using mice engrafted with two xenograft leukemias
The plasma levels of huIL-4 achieved in SCID mice with continuous infusion huIL-4 200 μg/kg/d were between 0.96 and 1.98 ng/mL. These values for effective in vivo concentrations were in agreement with the observed inhibition of xenograft leukemia cells in vitro by huIL-4 0.5 ng/mL. A previous experiment using huIL-4 100 μg/kg as a daily bolus subcutaneous injection to treat mice engrafted with ALL1 failed to show any beneficial effect of huIL-4 in terms of either levels of circulating ALL cells or survival (data not shown). With daily bolus injections high peak levels of huIL-4 were achieved, but within 4 hours had fallen below 0.5 ng/mL. This suggests that continuous exposure to 0.5 to 1.0 ng/mL concentrations of huIL-4 may be necessary to suppress leukemia in SCID mice, and further suggests that a continuous infusion of huIL-4 may be the appropriate schedule for the treatment of patients with ALL.

Xenograft leukemia cells obtained from huIL-4–treated animals continued to show inhibition by huIL-4 in vitro, although for both leukemias sensitivity had diminished. The continued in vitro inhibition raises the possibility of re-treating or prolonging treatment with huIL-4; however, the reduction in sensitivity suggests that huIL-4 alone at the concentrations achieved may not be curative in this model of ALL. The mechanism of huIL-4 inhibition of human ALL in SCID mice is unclear. In some situations IL-4 activity is mediated through the recruitment of murine effector cells. In a murine model, solid tumor growth was inhibited by enhanced host immunity when murine IL-4 was injected locally or when the murine IL-4 gene was transfected into tumor cells. However, human IL-4 and murine IL-4 share only a 49% homology of amino acid sequence and the activities are highly species specific. Furthermore, we have demonstrated huIL-4 inhibition of in vitro cultures of xenograft cells from mouse spleen that contain very few murine cells (<5%). Human IL-4 has been shown to induce apoptosis in lymphoblasts in vitro, and this may be a mechanism of action of huIL-4 against xenograft ALL. In humans, these...
direct effects of huIL-4 may be bolstered by stimulation of effector cells of the human immune system by the cytokine.

Human IL-4 has been shown to inhibit a range of human solid malignancies in vitro26,27 and several studies of IL-4 used in patients with solid tumors have been reported, with some favorable tumor responses.28-33 This is the first demonstration of activity of huIL-4 against primary human leukemia cells grown in SCID mice. These data suggest a use for IL-4 in the treatment of patients with ALL.

As with many cytokines, huIL-4 has a range of side effects when used to treat patients. Flu-like symptoms predominate at low-dosage levels and are generally not dose-limiting. However, at doses of 4 to 5 μg/kg/d headache, neurological disturbances, and alterations in liver function may occur whether IL-4 is administered by subcutaneous injectionz8 or IV infusion (Schering-Plough; data on file). Higher daily doses, above 5 μg/kg/d, have been tolerated in some studies, although some patients receiving high doses of IL-4 encountered problems of gastrointestinal hemorrhage and cardiac ischemia, which may have been related to the cytokine.

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