Inhibition of Growth of Mouse Leukemic Cell Lines in Vitro and in Vivo by a Monoclonal Antibody That Recognizes an Interleukin 3 Receptor-associated Protein

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ABSTRACT

Myeloid cell lines that have achieved leukemic transformation may also have acquired the ability to produce hematopoietic growth factors. In certain instances, neutralizing antibodies directed against the growth factor have inhibited growth, supporting an autocrine mechanism in their transformation. The effect of anti-receptor antibodies on their growth and transformed phenotype has not been reported. We have developed a monoclonal antibody, 4G8, directed against a protein that is associated with the IL-3 receptor complex; 4G8 inhibits IL-3 binding and signal transduction in nonleukemic IL-3-dependent cell lines. In this study, we examined the effect of 4G8 on the growth in vitro and in vivo of leukemic cell lines, including WEHI-3B, which spontaneously produces IL-3, and NFS-60, an IL-3-dependent cell line. Our results demonstrate that the 4G8 antigen can be detected in both WEHI-3B and NFS-60 cells by flow cytometry and Western blotting; 4G8 inhibits the autonomous growth of WEHI-3B and the IL-3-dependent growth of both WEHI-3B and NFS-60. In addition, s.c. administration of 4G8 prolonged the survival of syngeneic mice given s.c. injections of WEHI-3B. These results support the conclusion that an autocrine mechanism involving IL-3 and its receptor plays a critical role in the growth and transformed phenotype of WEHI-3B and raises the possibility that anti-IL-3 receptor antibodies may be useful in the treatment of some leukemias.

INTRODUCTION

IL-3 is a M, 28,000 myeloid growth factor produced by in vitro stimulated cells of a limited number of lineages that include T-lymphocytes, keratinocytes, mast cells, and natural killer cells (1–3). This growth factor exerts a pluripotent effect on bone marrow precursor cells producing multiple-lineage colonies in vitro and expanding the pool of early progenitor cells in vivo (1). Whereas the majority of myeloid precursor and mature cells possess receptors for IL-3, an in vivo bone marrow source of IL-3 remains elusive, rendering uncertain the role of IL-3 in the maintenance of normal hematopoiesis.

Like their normal counterparts, murine and human myeloid leukemia cells display receptors for myeloid growth factors including IL-3, and many have been shown to be responsive to exogenous IL-3 in vitro (4–6). Production of IL-3 has been demonstrated in some transformed myeloid cells, raising the possibility that autocrine production of IL-3 may have contributed to their transformed phenotype (7–9). The ability of neutralizing anti-IL-3 antibody to inhibit the growth of one IL-3-producing transformed cell line supports this conclusion (10). Additional support for an autocrine mechanism involving IL-3 and its receptor in IL-3-producing transformed cells might be provided by studies using anti-IL-3 receptor antibodies if such antibodies were available.

We have recently developed a monoclonal antibody, 4G8, that recognizes a surface protein with a molecular weight of 115,000–145,000 that is associated with the murine IL-3 receptor (11). This antibody blocks IL-3-mediated proliferation of nontumorigenic, bone marrow-derived, IL-3-dependent cell lines. We wanted to examine the effects of this antibody on the growth of WEHI-3B, an autonomously growing myeloid leukemic cell line that constitutively secretes IL-3, and on the growth of NFS-60, a leukemic cell line that requires exogenous IL-3 or G-CSF for growth. We demonstrated that both cell lines expressed moderate to high levels of 4G8 antigen, that the constitutive proliferation of WEHI-3B was inhibited by 4G8, and that 4G8 inhibited the IL-3-dependent proliferation of NFS-60. We also demonstrated that exogenous IL-3 augmented the growth of WEHI-3B; the exogenously induced growth of WEHI-3B was also inhibited by 4G8. Finally, the survival of mice given s.c. injections of WEHI-3B was prolonged by s.c. administration of 4G8.

MATERIALS AND METHODS

Cell Line. The mouse myelomonocytic leukemia cell line WEHI-3B (12) was maintained in IMDM supplemented with 1% FCS, 2 mM L-glutamine, penicillin, and streptomycin, and 2.5% W3CM. The IL-3-dependent myeloid leukemia cell line, NFS-60 (13), was maintained in IMDM with 10% FCS, 10% W3CM, 10 mM N-2-hydroxyethyl-piperazine-N'2-ethanesulfonic acid, 2 mM L-glutamine, penicillin, and streptomycin. The T-cell line EL-4 was obtained from Dr. Scott Cairns (Pittsburgh Cancer Institute) and grown in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, penicillin, and streptomycin.

Monoclonal Antibodies. The 4G8 hybridoma was established as described (11). J11.10, a rat IgM anti-mouse Thy1.2 hybridoma, was purchased from American Type Culture Collection. Conditioned medium from each hybridoma was prepared and partially purified as described (11).

Western Blotting. Western blotting was performed as described (11). Briefly, cells were lysed in nonreducing sample buffer and separated by sodium dodecyl sulfate on a 5–10% gradient gel. One million cells were loaded in each lane. The separated proteins were transferred onto nitrocellulose and, after blocking with solubilized powdered milk, the membrane was incubated with 4G8 antibody at 10 μg/ml, followed by incubation with an iodinated goat anti-rat IgM (Cappel, West Chester, PA). After three 20-min washes, the membrane was dried and autoradiographed.

Fluorescence Staining and Flow Cytometric Analysis. WEHI-3B cells were incubated with either medium alone or antibodies for 30 min at 4°C, washed, then incubated for 30 min at 4°C in FITC-conjugated mouse anti-rat IgM monoclonal antibody (Cappel). After washing, cells were analyzed on a FACStar flow cytometer (Becton Dickinson, San Jose, CA).

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3The abbreviations used are: IL-3, interleukin 3; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; W3CM, WEHI-3B conditioned medium; G-CSF, granulocyte-colony stimulating factor; PBS, phosphate-buffered saline; GM-CSF, granulocyte-macrophage-colony stimulating factor; T-ALL, T-acute lymphocytic leukemia.
Partial Purification of IL-3 from WEHI-3B. WEHI-3B cells were grown to 50% confluence, transferred to serum-free media, and incubated for 1 week. Conditioned medium was filter-sterilized to remove cellular debris and concentrated 20-fold by filtration through a PM-30 Amicon membrane (Amicon, Danvers, MA), then applied to a Mono-Q anion-exchange column (Pharmacia, Piscataway, NJ) and eluted with a NaCl gradient (0 to 0.7 M). Fractions of 3 ml were collected and tested for their ability to induce proliferation of 32Dcl3 cells in a [3H]-thymidine incorporation assay. Active fractions were pooled and the IL-3 activity of the pooled fractions was determined by titration in a [3H]thymidine incorporation assay along with recombinant murine IL-3 (Genzyme, Boston, MA).

Clonogenic Assay. WEHI-3B, NFS-60, or EL-4 cells were suspended in 1 ml of IMDM containing 0.3% Seaplaque agarose (Cuno, Farmington, CT), FCS, L-glutamine, penicillin, and streptomycin at a cell concentration of 10^6/ml and plated into 35-mm Petri dishes in duplicates. Dishes were incubated in a humidified chamber in a CO2 incubator and colonies were counted after 7 days. Cell aggregates containing 40 or more cells were counted as a colony.

In Vitro Antitumorigenic Assay. The WEHI-3B tumor model (14) was used to test the efficiency of 4G8 in vivo. Briefly, for each experiment, 15 healthy, 12-week-old female BALB/c mice were given s.c. injections of 5 \times 10^6 WEHI-3B cells into one flank on day 0. They were divided into 3 groups. Group 1 received once-daily s.c. injections of PBS (0.5 ml) into the flank opposite the tumor injection site daily on days 1 through 4. Groups 2 and 3 each received partially purified Jlj.10 (0.5 ml of 1 mg/ml) or 4G8 (0.5 ml of 1 mg/ml), respectively, into the flank opposite the tumor injection site daily on days 1 through 4. The animals were maintained in a pathogen-free animal facility and observed daily until their death.

Statistical Analysis. In the analysis of the dose-response effects of antibodies, the curves were fitted by linear regression and compared using the dummy-variable model (15).

RESULTS

Flow Cytometric and Western Immunoblot Analysis. We have previously demonstrated that 4G8 stains all nonleukemic IL-3-dependent cell lines tested including 32Dcl3, 32Dcl5, MC9, MCvl.8, and PT18 (11). Both NFS-60 and WEHI-3B stained brightly with 4G8 (Fig. 1). Levels of expression of the 4G8 antigen in NFS-60 and WEHI-3B were similar to that of nonleukemic cell lines. Western blot analysis of NFS-60 and WEHI-3B cells demonstrated a single band at Mr 110,000 and 127,000, respectively, similar to the size of this antigen in nonleukemic cells (Fig. 2).

Effect of 4G8 on the Proliferation of NFS-60 Cells. NFS-60 is a leukemia cell line derived from a tumor induced by the wild mouse ecotropic virus (Cas BrM murine leukemia virus) (13) that is IL-3 dependent for growth. In the presence of a suboptimal concentration of IL-3 (10 units/ml), 4G8 inhibited the [3H]thymidine incorporation of NFS-60 in a dose-dependent fashion (Fig. 3A; P < 0.025, linear regression analysis). The proliferation of cells incubated with Jlj.10 was not affected. Jlj.10 is a rat IgM monoclonal antibody directed against mouse Thy1.2 that stains NFS-60 with similar intensity. Similar to its effect on nonleukemic IL-3-dependent cell lines (11), the inhibition by 4G8 was incomplete, achieving a maximum of 40% at an antibody concentration of 3 \mu g/ml. The effect of 4G8 on NFS-60 colony formation was more striking (Fig. 3B), approaching 70% inhibition at 3 \mu g/ml antibody concentration, whereas Jlj.10 again had little to no effect. The effect of 4G8 on NFS-60 was specific for the IL-3 pathway since the proliferation of these cells induced by G-CSF was not affected by 4G8 (data not shown).

Effect of 4G8 on the Proliferation of WEHI-3B Cells in Vitro. WEHI-3B is a subline of WEHI-3, a myelomonocytic leukemic cell line that constitutively produces IL-3 (12). 4G8 inhibited the spontaneous [3H]thymidine incorporation of WEHI-3B in a dose-dependent fashion (Fig. 4A; P < 0.025, linear regression analysis) achieving 60% inhibition at 3 \mu g/ml whereas Jlj.10 had no effect. To confirm the antiproliferative effect, colony assays were performed; 4G8 had a striking inhibitory effect on WEHI-3B colony formation (Fig. 4B; P < 0.05, linear regression analysis) but did not effect the colony formation of EL4, a thymoma cell line that stains equally intensely with 4G8.

To confirm that 4G8 inhibited the growth of WEHI-3B through an IL-3-dependent mechanism, we examined whether the growth of WEHI-3B can be augmented by exogenous IL-3 and whether this augmented growth could be inhibited by 4G8. In colony assays performed under low serum conditions (1% FCS), exogenous IL-3 increased the cloning efficiency of WEHI-3B over 3-fold (Fig. 5A). The IL-3-enhanced cloning...
demonstrated a distinct shift, indicating that WEHI-3B cells express low levels of the low-affinity IL-3 receptor. The failure by others to detect high-affinity binding sites using radioligand binding studies, or by us using biotinylated IL-3, may be a result of autologous production of IL-3 leading to down-modulation of high-affinity sites. Since the number of low-affinity sites exceeds the number of high-affinity sites by 10–100-fold (18),

**Fig. 2.** Western immunoblotting of EL4 (Lane 1), WEHI-3B (Lane 2), and NFS-60 (Lane 3), and the B-cell line, M12 (Lane 4) using 4G8. Cell lysates were electrophoresed, blotted, and incubated with 4G8 as described in “Materials and Methods.” The membrane was exposed 21 h before developing.

efficiency could be inhibited by 4G8 in a dose-dependent fashion (Fig. 5B), supporting the conclusion that 4G8 is inhibiting the proliferation of WEHI-3B by blocking IL-3 signal transduction.

In our previous study (11), we postulated that 4G8 antigen is associated with the IL-3 receptor complex on the cell surface. When WEHI-3 was examined for high-affinity IL-3 binding sites using radiolabeled IL-3 (16), no binding sites were found. Using biotinylated IL-3 followed by avidin-FITC, we were able to demonstrate high-affinity IL-3 binding sites on 32Dcl3 and NFS-60 but not on WEHI-3B (data not shown). Itoh et al. (17) have recently cloned the low-affinity IL-3 receptor using the monoclonal antibody AiC2 in an expression cloning system. We examined WEHI-3B and NFS-60 by flow cytometry for the expression of the low-affinity IL-3 receptor using AiC2 (Fig. 6). Although expressing a substantially lower density of AiC2 antigen than NFS-60, WEHI-3B cells incubated with AiC2

**Fig. 3.** Inhibition of IL-3-induced proliferation of NFS-60 by 4G8. A, 10⁴ cells were incubated in microtiter wells containing IL-3 (10 units/ml) with or without 4G8 (DH) or Jlj.10 (*-*) antibody at the indicated concentrations. After 48-h incubation, each well was pulsed with 1 μCi [³H]thymidine, harvested 18 h later, and counted. The data plotted are the mean ± SD of triplicate wells. B, 10⁴ cells were incubated in IL-3 (10 units/ml) and 0.3% agarose in 30-mm Petri dishes with or without 4G8 (DH) or Jlj.10 (*-*) antibody at the indicated concentrations. After 7-day incubation, colonies (>40 cells) were counted. The data plotted are the mean ± SD of duplicate plates.

**Fig. 4.** Inhibition of spontaneous WEHI-3B proliferation by 4G8. A, 10⁴ cells were incubated for 48 h in microtiter wells with or without 4G8 (DH) or Jlj.10 (*-*) antibody at the indicated concentrations. Cells were pulsed and harvested as outlined in Fig. 3. The data plotted are the mean ± SD of triplicate wells. B, 10⁴ WEHI-3B (DH) cells or EL4 (*-) cells were incubated with 4G8 at the indicated concentrations in 0.3% agarose in 30-mm Petri dishes. After 7 days, colonies (>40 cells) were counted. The data plotted are the mean ± SD of duplicate plates.

**Fig. 5.** Effect of IL-3 alone or IL-3 plus 4G8 on WEHI-3B colony formation. A, 10⁴ WEHI-3B cells were incubated in 0.3% agarose in 3.0-mm Petri dishes with or without IL-3 at the concentrations indicated. Data plotted are the mean ± SD of triplicate plates. B, 10⁴ WEHI-3B are incubated in 0.3% agarose containing 10 units/ml IL-3 with or without 4G8 at the concentrations indicated. Data plotted are the mean ± SD of triplicate plates.
it may not be surprising that some low-affinity sites are detectable after all high-affinity sites are down-modulated.

Prolongation of Survival of Syngeneic Mice Given Injections of WEHI-3B. The results of the in vitro studies suggested that 4G8 could be of therapeutic benefit in an experimental tumor model using WEHI-3B. To test this possibility, 3 groups of 5 mice injected s.c. with WEHI-3B cells were given 4 consecutive daily s.c. injections of PBS, 4G8, or J11.10 in the flank, as described in “Materials and Methods.” Injection of 4G8 prolonged median survival by 18% compared with the J11.10 group (Fig. 7; P < 0.05, log-rank test).

DISCUSSION

We have demonstrated that a rat monoclonal antibody, 4G8, which identifies a protein associated with the IL-3 receptor complex, brightly stains WEHI-3B and NFS-60 and identifies a band by Western immunoblotting of M, 127,000 and 110,000, respectively. Similar to its effects on nonleukemic IL-3-depend-
through its inhibitory effect on IL-3 signal transduction and not through antibody- and complement-mediated cytotoxicity. This conclusion is supported by the demonstration that treatment of WEHI-3B-injected mice with J11.d10 using the same regimen was without effect. Like 4G8, J11 is a rat anti-mouse IgM monoclonal antibody and it stains WEHI-3B with equal intensity.

Waldman et al. (21) have successfully used monoclonal antibodies against the alpha-chain of the IL-2 receptor (anti-Tac antibodies) to achieve limited, partial, or complete remissions in 3 of 9 patients with human T-cell leukemia virus-1-induced adult T-cell leukemia. The treatment rationale included the fact that anti-Tac antibodies inhibit IL-2-dependent proliferation of T-cells. Adult T-cell leukemia cells express high levels of Tac antibodies against the alpha-chain of the IL-2 receptor (anti-Tac antibodies) while the vast majority of normal T-cells would not be affected.

Although a few human acute myeloid leukemias express IL-2 receptors (22), most express IL-3 receptors (4) and proliferate in response to IL-3 (4–6). Several murine leukemia cell lines, like WEHI-3B, may have arisen, in part, through autocrine production of IL-3 (7–9). Production of IL-3 by human leukemic cells has been demonstrated occasionally in acute myeloid leukemia and has been observed in 7 of 17 cases of T-ALL (23). In addition, low level expression of IL-3 receptors has been demonstrated in 2 of 5 T-ALL samples by Park et al. (4). Although these investigators did not examine the ability of the T-ALL samples to proliferate in response to IL-3, we have established a GM-CSF-dependent cell line, TALL-101, from a child with T-ALL, that could also be maintained in IL-3 (24, 25), suggesting that some T-ALL cells proliferate in response to IL-3.

Antibodies effective at blocking signal transduction through the IL-3 receptor may be useful in treating acute myeloid and lymphoid leukemia similar to anti-Tac antibodies in a human T-cell leukemia virus-1-induced leukemia. While most normal myeloid cells express IL-3 receptors, the failure to find a bone marrow source of IL-3 suggests that IL-3 may not be critical for normal hematopoiesis. Consequently, antibodies that block IL-3 signal transduction in acute leukemia cells and inhibit their proliferation may not be detrimental to normal hematopoiesis. In vitro experiments in our laboratory indicate that while 4G8 inhibits IL-3-induced colony formation, it enhances colony formation induced by GM-CSF, G-CSF, and macrophage-CSF.4 Experiments to examine the effect of 4G8 on hematopoiesis in vivo and to extend the observed antileukemic effect to other IL-3-producing leukemic cell lines are under way.

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