Interleukin-9 Is a Major Anti-Apoptotic Factor for Thymic Lymphomas

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We have recently shown that interleukin-9 (IL-9) strongly stimulates the proliferation of mouse thymic lymphomas in vitro. Here we report that this factor is also one of the most potent inhibitors of apoptosis induced by dexamethasone (DEX) in such cell lines, even if they do not depend on exogenous factors for growth. For the murine thymic lymphoma BWS147, protection against apoptosis was also obtained with IL-4 and less strongly with IL-6, whereas IL-2, IL-7, and IL-10 were inactive. Moreover, IL-4 and IL-9 maintained proliferation of these cells in the presence of DEX. Analysis of eight other factor-independent thymic lymphoma lines showed significant protection in seven and six cell lines with IL-9 and IL-4, respectively, whereas only three were protected by IL-7 and only two by IL-2. Comparison of the responses to IL-2 and IL-9 in a factor-dependent cell line that responds to both cytokines showed that IL-2 is a stronger inducer of proliferation, while IL-9 is more efficient in protecting the cells against apoptosis. Taken together, our observations suggest that, for thymic lymphomas, proliferation and apoptosis involve distinct regulatory mechanisms and can be differentially regulated by cytokines.

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MATERIALS AND METHODS

Tumors and cell lines. Thymic lymphoma cell lines BWS147 and WEHI 22.1 were obtained from the American Type Culture Collection (Rockville, MD). TH1821 was derived from a γ-ray-induced CBA/H thymic lymphoma. The other cell lines were established in our laboratory (Ludwig Institute for Cancer Research, Brussels, Belgium): NM2TI and NM5T2 from DBA-2 mice treated with N-methyl-N-nitrosourea (MNU); BR14T2 from a C57BL/6 mouse submitted to fractional x-ray irradiation, and 9T4A2 and 9T7 from thymic lymphomas that spontaneously arose in IL-9-transgenic mice but had lost the IL-9 transgene. Cell line 9T7.9C1 is an autonomous subclone from the IL-9-dependent 9T7 lymphoma. All cells were cultured in Iscove’s medium supplemented with 10% fetal calf serum, 1.5 mmol/L L-glutamine, 0.24 mmol/L 2-mercaptoethanol.

Cytokines and other reagents. The following cytokines were provided as indicated: human recombinant IL-1α from Dr P. Lomedico (Roche Research Center, Nutley, NJ); human IL-2, mouse interferon-γ (IFN-γ), and mouse tumor necrosis factor-α (TNF-α) from Dr W. Fiers (State University of Ghent, Ghent, Belgium); human recombinant IL-7 from R & D Systems (Minneapolis, MN); mouse IL-10 from Dr P. Vieira and K.W. Moore (DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, CA); and mouse IL-13 from Dr J. Banchereau (Schering-Plough, Dardilly, France). Murine IL-4, IL-6, and IL-9 were produced by expression in baculovirus and purified by affinity chromatography at Ludwig Institute for Cancer Research. DEX was used at 0.25 μmol/L concentration in all experiments. Staurosporin (Biomol Research Laboratories Inc, Piscataway, NJ) was used at 0.1 μmol/L.
of DEX, thymic lymphoma cell lines were incubated overnight in triplicate microtiter wells with or without cytokines before addition of DEX to a final $0.25 \times 10^{-8}$ mol/L concentration in a 200-$\mu$L volume. Unless stated otherwise, the cytokine concentrations used were as follows: IL-1$, 20 \text{ U/mL}$; IL-2, 50 $\text{ U/mL}$; IL-4, 250 $\text{ U/mL}$; IL-6, 1,000 $\text{ U/mL}$; IL-7, 10 $\text{ ng/mL}$; IL-9, 500 $\text{ U/mL}$; IL-10, 10 $\text{ ng/mL}$; IL-13, 100 $\text{ ng/mL}$; IFN$\gamma$, 100 $\text{ U/mL}$; and TNF$\alpha$, 100 $\text{ U/mL}$. These concentrations were at least 10-fold higher than doses giving a maximal activity of the factors in their respective bioassays. Cultures were pulsed 2 to 4 days later for 6 hours with $0.5 \mu\text{Ci}$ $\text{H}$-thymidine. Alternatively, cell proliferation was measured by an hexosaminidase assay, as described.\textsuperscript{7}

### RESULTS

**IL-9 inhibits DEX-induced apoptosis in BW5147 thymic lymphoma cell line.** IL-9 is a potent stimulator of thymic tumor cell proliferation, but its ability to inhibit apoptosis has not yet been investigated. To evaluate possible antiapoptotic activity mediated by IL-9, we selected BW5147, a thymic lymphoma that proliferates actively in vitro without cytokines and undergoes apoptotic cell death in the presence of DEX. Cells incubated with or without a saturating dose of IL-9 were exposed to DEX for 30 hours. Genomic DNA was extracted and analyzed by gel electrophoresis to detect fragmentation into 200-bp fragments as a reflection of apoptosis. Cell viability was also measured by FACS after staining with propidium iodide. As indicated in Fig 1, exposure to DEX induced a strong DNA fragmentation and approximately 30% cell death after 30 hours. Both processes were almost completely inhibited by IL-9 (Fig 1). Pretreatment of BW5147 with IL-9 was, in fact, not necessary, as cells were protected as efficiently when the cytokine was added at the same time as DEX (data not shown).

**Activity of other cytokines in protecting BW5147 against DEX-induced apoptosis.** To evaluate the antiapoptotic activity of other cytokines, BW5147 cells were incubated with a series of different factors before addition of DEX. Cell mortality was measured by propidium iodide staining after

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**Fig 1. Inhibition by IL-9 of DEX-induced apoptosis in BW5147 cells.** (A) Electrophoresis of DNA extracted from BW5147 cells cultured for 3 days in control medium or in the presence of IL-9 (500 $\text{ U/mL}$). DEX (0.25 $\mu\text{mol/L}$) was added for the last 30 hours of the culture. (B) Propidium iodide staining and FACS analysis of BW5147 cells cultured for 24 hours in control medium, in DEX, or in DEX and IL-9. Y axis corresponds to the cell numbers and X axis, to fluorescence intensity. Living cells, which showed no DNA staining, were differentiated from dead cells, whose DNA was stained.

**Fig 2. Cytokines protecting BW5147 cells against DEX-induced apoptosis.** Cells were incubated for 20 hours with saturating concentrations of the indicated cytokines, as described in Materials and Methods, before addition of DEX (0.25 $\mu\text{mol/L}$). Cells were stained 24 hours later with propidium iodide, and the proportion of positive cells was measured by FACS for duplicate cultures. In the absence of DEX, the mean proportion of stained cells was 2.5% ± 1.4%. The results are representative of three separate experiments.
a 24-hour exposure to DEX. This analysis showed that, IL-4 and IL-6, in addition to IL-9, also inhibited DEX-induced mortality, whereas IL-1, IL-2, IL-7, and IL-10 were inactive. By contrast, TNF-α and, to a lesser extent, IFN-γ increased DEX-induced mortality (Fig 2). Interestingly, IL-9 also inhibited the increment in mortality induced by these factors (data not shown).

As inhibition of DEX-induced apoptosis does not necessarily result in cell proliferation, we evaluated the growth-stimulatory activity of cytokines on BW5147 cells in the presence of DEX. With DEX alone, the proliferation was inhibited by 90% to 95% (data not shown). By contrast, in the presence of IL-9, a strong proliferation was observed in thymidine incorporation or hexosaminidase assays. Lower but significant levels of protection were obtained with IL-4 and IL-6, whereas other cytokines, such as IL-1α, IL-2, IL-10, and IL-13, were inactive (Fig 3). Concentrations of IL-4 required to maintain proliferation were much higher than those of IL-9. Half-maximal protection required 500 pmol/L for IL-4, while the same result was obtained with 5 pmol/L IL-9, this sensitivity making BW5147 in the presence of DEX equivalent to the most sensitive biologic assay for IL-9 described so far. For IL-6, the half-maximal effect was obtained at a concentration on the order of 10 pmol/L, which is similar to that which induces half-maximal responses in fresh mouse T cells9 (Fig 4).

To test whether the protective activities of IL-4 and IL-9 against DEX were long-lasting, cell cultures were established with or without DEX and successively diluted for 2 weeks. As shown in Fig 5, the protective activities of either factor allowed for permanent growth in the presence of DEX. Over a 12-day period, the number of divisions was 39 in the absence of DEX and 29 or 26 with DEX and IL-9 or IL-4, respectively. With DEX in the absence of cytokines, viable cells could no longer be detected after 1 week. Without DEX, cytokines do not affect cell growth (data not shown).

Effect of staurosporin and CsA on rescue mechanisms triggered by cytokines in DEX-treated BW5147 cells. To begin to analyze the signal transduction mechanisms involved in the anti-apoptotic activity of IL-9, IL-4, and IL-6, we evaluated the effect of staurosporin (an inhibitor for a large variety of kinases) and CsA. Although these reagents did not affect cell viability in normal medium, they significantly accelerated DEX-induced cell death. In the presence of 5 ng/mL of staurosporin, the activities of IL-4 and IL-9 were completely blocked, while IL-6 had still a weak but reproducible activity. By contrast, CsA at 1 μg/mL did not affect the protection conferred by IL-4 or IL-9 but significantly reduced the effect of IL-6 (Fig 6). These results suggest that distinct signaling pathways are activated by IL-9 and IL-4 on the one hand and IL-6 on the other.

Activity of T-cell growth factors against DEX-induced apoptosis of other thymic lymphoma cell lines. The ability of IL-2, IL-4, IL-7, and IL-9 to prevent apoptosis was examined on eight other factor-independent, DEX-sensitive thymic lymphoma cell lines obtained under distinct conditions in different mouse strains. These cells were incubated with
or without cytokines for 24 or 48 hours before addition of DEX. Significant inhibition (at least 20%) of DEX-induced cell death was observed in seven cell lines with IL-9, in six with IL-4, in three with IL-7, and in two with IL-2 (Fig 7). However, reversal of the antiproliferative activity of DEX by cytokines was not observed in all thymic lymphoma cell lines where significant inhibition of apoptosis had been detected: in three of the seven cell lines tested, we did not detect significant growth in the presence of DEX with IL-2, IL-4, IL-7, or IL-9 (Table 1). The failure of cytokines to maintain proliferation in the presence of DEX in these cells contrasted with their strong anti-apoptotic activities observed with the same cells, indicating that protection against apoptosis does not automatically correlate with cell proliferation.

To compare the efficiency of IL-9 and IL-2 to induce proliferation and to protect against apoptosis, we used the lymphoma cell line 9T7, which proliferates autonomously but whose growth can still be stimulated by IL-2. In the absence of DEX, IL-9 stimulates 9T7 proliferation only poorly, whereas IL-2 induces a 100% increase in thymidine incorporation. By contrast, in the presence of DEX, IL-9 was found to be more efficient than IL-2 to protect cells against apoptosis (35% v 17% of cytokine-induced protection). Moreover, when IL-9 and IL-2 were combined, additive effects were observed both for proliferation and protection against apoptosis (Fig 8), further suggesting that the mechanisms involved in the activities of these cytokines are distinct.

**DISCUSSION**

The results presented here show that IL-9 is a potent inhibitor of glucocorticoid-induced apoptosis in thymic lymphoma cell lines that do not depend on cytokines for growth. For the BW5147 lymphoma cell line, IL-4 was an equally potent inhibitor of apoptosis as IL-9, but with a lower specific activity. IL-6 also reduced DEX-induced cell death, but less than the two other factors. Attempts to prevent the anti-apoptotic activity of IL-9, IL-4, and IL-6 with staurosponin or CsA showed a definite difference: whereas the anti-apoptotic activity of IL-4 and IL-6 was inhibited by staurosponin and not by CsA, the converse was true for IL-9. The opposite effects of these two reagents suggest that the anti-apoptotic activity of IL-6 involves different mechanisms than those triggered by IL-4 and IL-9. Comparison of the anti-apoptotic properties of various cytokines on different cell lines indicates that IL-4 and IL-9 give the best protection against DEX. Protection was also observed, but less frequently, with IL-7, while IL-2 was poorly active in this kind of assay.

The strong inhibition by IL-9 of DEX-induced apoptosis in thymic lymphoma cell lines contrasts with the absence of proliferative activity observed with freshly isolated thymocytes and further shows the preferential action of IL-9 on activated or transformed T cells. Recent observations have
indeed shown that resting human T cells are clearly unresponsive to IL-9 but become responsive soon after in vitro preactivation. The fact that thymic progenitors similarly acquire IL-9 responsiveness upon transformation was previously reported for freshly isolated tumoral samples. Moreover, we found recently that constitutive IL-9 expression in vivo does not affect the normal thymic development but results in an abnormally high incidence of thymic lymphomas. The present demonstration that IL-9 is particularly efficient in the inhibition of apoptosis, in addition to its ability to stimulate cell growth, provides a potential explanation for these results, as apoptosis is a characteristic of proliferating cells.

Contrary to IL-9, IL-2 exhibits a poor anti-apoptotic activity on the thymic lymphoma cell lines used in this study. A simple explanation for this observation would be the loss of IL-2 receptor-α expression by some tumors. Nevertheless, the levels of protection against DEX provided by IL-2 on cells that express functional IL-2 receptors are rather week as compared with those conferred by IL-4 or IL-9. Moreover, when we used the 9T7 lymphoma cell line, where IL-2 receptor-α was expressed and whose spontaneous proliferation rate could still be enhanced by IL-2, only minimal protection against DEX was obtained with this factor. IL-9, on the contrary, had a negligible stimulatory effect on cell growth but inhibited apoptosis more clearly. The fact that IL-9 strongly protected several cell lines against apoptosis while failing to maintain cell proliferation further suggests that distinct regulatory elements are involved in these two processes.

Interestingly, with 9T7 cells, combination of IL-2 and
IL-9 showed an additive activity both for proliferation and protection against apoptosis. In another cell line, proliferation in the presence of DEX required the combination of IL-2 and IL-9 (data not shown), further demonstrating the synergistic interaction between IL-2 and IL-9 originally observed for the proliferation of fetal thymocytes. In light of these results, an attractive explanation for this synergy might be that IL-2 and IL-9 act preferentially on two distinct and complementary aspects of cell growth: cell division for IL-2 and cell survival for IL-9.

The present study indicates that the putative role of cytokines, and particularly of IL-9, in the development of thymic lymphomas is not limited to cells that depend on these factors for proliferation. This possibility enlarges the spectrum of activities of these molecules with respect to tumors that are sensitive to inducers of apoptosis in vivo. The importance of this function has been clearly demonstrated by the finding that IFN-γ, which is constitutively produced in B-cell chronic lymphocytic leukemia, protects these cells against apoptosis without stimulating their proliferation. Further studies will have to assess the hypothesis of a similar role for IL-9 in lymphoid malignancies in which an IL-9 autocrine loop has been suggested.

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