Brief Definitive Report

INHIBITION OF HUMAN MACROPHAGE COLONY FORMATION BY INTERLEUKIN 4

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IL-4 was initially described as a co-stimulant of DNA synthesis for B lymphocytes stimulated with anti-IgM antibody (1). Since then, many effects of IL-4 on various hematological cell types have been reported (for review see reference 2).

The effects of IL-4 on hematopoiesis have not been studied extensively. On both murine (3, 4) and human (5) bone marrow cells, enhancement of erythroid colony growth by IL-4 in the presence of erythropoietin (EPO) has been reported. In addition, IL-4 enhances granulocyte colony-stimulating factor (G-CSF)-induced granulocytic colony formation (3–5).

In the present study we describe the effects of human rIL-4 on hematopoiesis in vitro. We show a marked, dose-dependent reduction of macrophage colony formation (CFU-M) after addition of IL-4 to bone marrow progenitors stimulated with either granulocyte/macrophage CSF (GM-CSF) or macrophage CSF (M-CSF).

Materials and Methods

IL-4. Human rIL-4 was kindly provided by Dr. P. Herman and Dr. P. P. Trotta, Schering Corp., Bloomfield, NJ. Human IL-4 c-DNA was expressed in Chinese hamster ovary (CHO) cells and the produced (glycosilated) protein was purified (>95% pure). For control experiments, a second IL-4 preparation was used (Genzyme, Boston, MA). The c-DNA for this IL-4 was expressed in yeast, enzymatically deglycosilated, and purified (>95% pure). This protein had a specific activity of 10^8 U/mg, 1 unit being defined as half-maximal stimulation in a B cell proliferation assay.

Anti-IL-4 Antiserum. Anti-IL-4 antiserum, a kind gift of Dr. J. E. de Vries, (Unicet, Dardilly, France) was raised in a rabbit against human rIL-4. This anti-IL-4 antiserum blocked the biological activity of IL-4 as measured in both a B and a T cell proliferation assay.

Cell Preparations. Bone marrow mononuclear cells were obtained after informed consent from donors for allogeneic bone marrow transplantation or from hernia nuclei pulposi patients who underwent laminectomy. Light density bone marrow cells were isolated by Ficoll Isopaque (1.077 g/cm^3) density gradient centrifugation. If necessary, the cells were depleted of monocytes by carbonyl iron incorporation and a magnetic stirrer, and they were T cell-depleted by rosetting with 2-aminoethylisothiouronium hydrobromide-treated sheep erythrocytes followed by Ficoll separation (6, 7). These depleted bone marrow suspensions contained <1% monocytes, as determined by α-naphthyl acetate esterase (ANAE) staining and <1% T cells as determined by staining with anti-CD3 mAb (anti-Leu-4; Becton Dick-

This work was partially supported by a grant from the Dutch Cancer Society. Address correspondence to J. H. Jansen, University Medical Center, Bldg. 1, C-2R, Rijnsburgerweg 10, 2333 AA Leiden, The Netherlands

inson & Co., Mountain View, CA) and goat anti-mouse FITC (GAM-FITC; CLB, Amsterdam, The Netherlands).

For some experiments, bone marrow cells were enriched for progenitor cells using mAbs against the CD34 and HLA-DR antigens, followed by FACS. Monocyte- and T cell-depleted light density bone marrow cells (2.5×10^6 cells/ml) were preincubated with 5% rabbit serum to prevent nonspecific Fc binding followed by incubation with 20 μg/ml anti-CD34 (MY10) mAb (Becton Dickinson & Co.). Cells were labeled with GAM-FITC (6 μg/ml) and incubated with Iscove's modified Dulbecco's medium (IMDM) containing 50% mouse serum in order to block nonspecific binding of the subsequent antibodies. Next, cells were incubated with 1.3 μg/ml anti-HLA-DR antibody conjugated to phycoerythrin (Becton Dickinson & Co.). $CD34^+HLA\text{-}DR^+$ cells were sorted using a FACStar (argon-ion laser tuned at 488 nm, Becton Dickinson & Co.). From the $HLA\text{-}DR^+$ cells, presenting as a separate cluster, the $CD34^+$ cells were isolated. The cut-off point was chosen after the first log decade, based on the fluorescence intensity of a control sample stained with the GAM-FITC-labeled antibody only.

BFU-E Cultures. Bone marrow progenitor cells were cultured as described (6, 7). Light density bone marrow cells were cultured at a concentration of 10^5/ml in IMDM containing 20% FCS, 5×10^{-5} M 2-ME, 0.5 g/liter deionized BSA, 0.47 g/liter human transferrin (Behringwerke AG, Marburg, Federal Republic of Germany), 10^{-5} $FeCl_3 \cdot 6H_2O$, and 0.98% methylcellulose (Methocel, Oud Beyerland, The Netherlands). Colony formation was stimulated with purified human EPO (1 U/ml; kindly provided by Organon Teknika BV, Turnhout, Belgium) and either human rGM-CSF (50 U/ml; a gift of the Schering Corp.), or human rM-CSF (a gift of Dr. P. Ralph; Cetus Corp., Emeryville, CA). 100 μl/well was plated in sixfold in flat-bottomed microtiter plates (Greiner, Alphen a/d Rijn, The Netherlands). Growth of the relatively mature erythroid colony forming units (CFU-E, compact clusters of 8–64 erythroid cells) was scored on day 7. The more immature erythroid burst forming units (BFU-E), granulocyte colony forming units (CFU-G, clusters of >50 granulocytes) and CFU-M (clusters of >20 macrophages) were scored on day 14.

CFU-GM Cultures. Monocyte- and T cell-depleted light density bone marrow cells were cultured at a concentration of 0.5×10^5 cells/ml. $CD34^+HLA\text{-}DR^+$ cells were cultured at a concentration of 10^4 cells/ml. Cells were cultured in α-MEM containing 20% FCS and 0.96% methylcellulose. Colony growth was stimulated with 50 U/ml human rGM-CSF. 100 μl/well was plated in sixfold in flat-bottomed microtiter plates. CFU-G and CFU-M colonies were scored on day 14.

Cultures were kept in a fully humidified 5% CO_2 atmosphere at 37°C. After the culture period, colonies were scored with an inverted microscope and, in some experiments, mass-harvested or picked individually with a micropipette, spread on microscope slides, and stained with ANAE or May-Grünwald Giemsa to confirm the monocytic or granulocytic lineage.

Results

Light Density Bone Marrow Cells. To study the influence of IL-4 on colony formation by human bone marrow progenitor cells, we added increasing concentrations of human rIL-4 to cultures of light density bone marrow cells stimulated with human rGM-CSF and EPO. A dose-dependent inhibition of macrophage colony formation was observed in bone marrow cultures of five successive donors (Fig. 1). At a concentration of 5 ng IL-4/ml, macrophage colony growth was reduced to ± 20% of control values. In contrast, erythroid (CFU-E and BFU-E) and granulocytic colony growth were not affected by IL-4.

Role of Accessory Cells. To study whether the IL-4-dependent inhibition of macrophage colony growth was mediated by accessory cell populations, we cultured monocyte- and T cell-depleted light density bone marrow cells stimulated with GM-CSF. Again, IL-4 induced a dose-dependent inhibition of macrophage colony formation (Fig. 2 *A*), suggesting that the effect was exerted directly on the hematopoietic

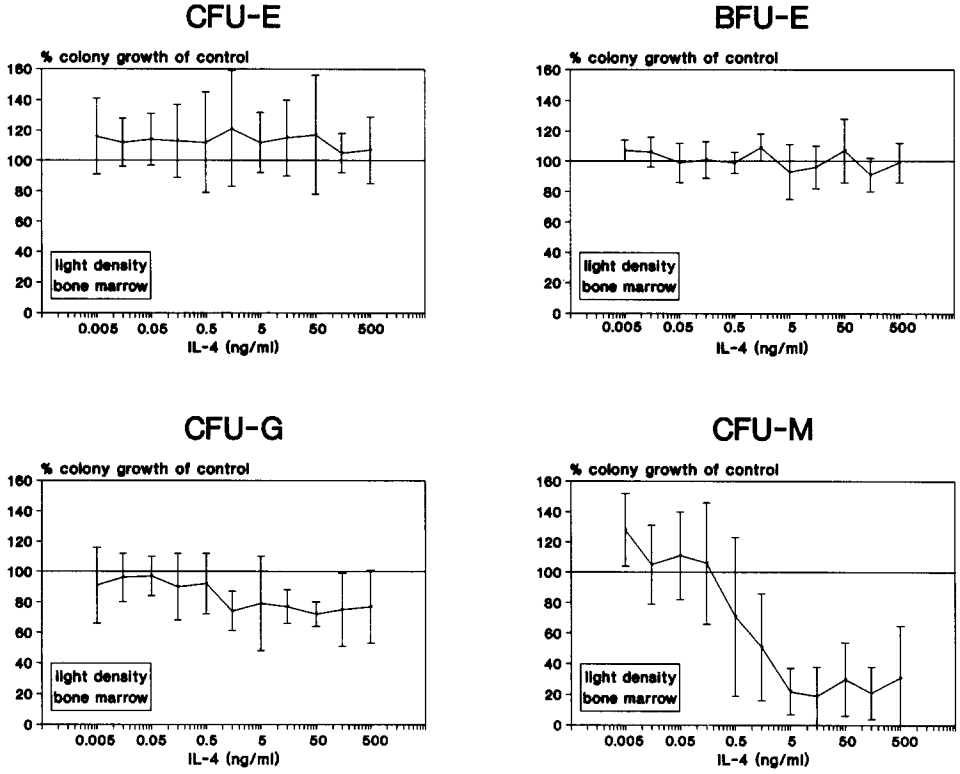

FIGURE 1. Influence of IL-4 on colony formation by light density bone marrow cells stimulated with GM-CSF (50 U/ml) and EPO (1 U/ml). Data are expressed as percentage (mean ± SD, n = 5) of the number of colonies obtained in control cultures (without IL-4 added). In these cultures, mean control growth was 106 CFU-E, 104 BFU-E, 76 CFU-G, and 55 CFU-M colonies/10^5 cells.

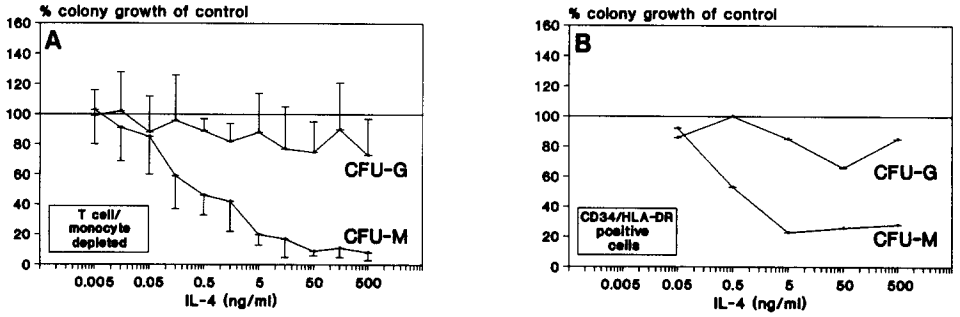

FIGURE 2. Influence of IL-4 on colony formation by (A) monocyte/T cell-depleted (n = 5, mean ± SD) and (B) $CD34^+/HLA\text{-}DR^+$-enriched bone marrow cells stimulated with GM-CSF (50 U/ml). Data are expressed as percentage of the number of colonies obtained in control cultures (without IL-4 added). In the monocyte/T cell-depleted cultures, mean control growth was 100 CFU-G and 269 CFU-M colonies/10^5 cells. Control growth in the $CD34^+/HLA\text{-}DR^+$ cultures was 2,483 CFU-G and 3,950 CFU-M colonies/10^5 cells.

progenitor cells. To further explore this, we cultured FACS-sorted $CD34^+HLA\text{-}DR^+$ cells stimulated with GM-CSF. A similar dose-dependent inhibition of macrophage colony formation by IL-4 was found, whereas granulocytic colony numbers were not reduced (Fig. 2 *B*).

Control Experiments. To confirm that the observed inhibition was due to IL-4 and not to a toxic contamination, we performed the following control experiments.

IL-4 was heat inactivated (20 min at 100°C) and added to the cultures at a concentration of 50 ng/ml. In all experiments (n = 8) the IL-4-induced inhibition of CFU-M was completely abrogated (data not shown).

Human rIL-4 from another source, a nonglycosilated protein, was tested on monocyte- and T cell-depleted bone marrow cells. This IL-4 had a similar inhibitory effect on CFU-M growth (inhibition up to 90%) as the recombinant human glycosilated IL-4 preparation, at the same concentration of IL-4 as measured in an ELISA, courtesy of Dr. J. E. de Vries (data not shown).

IL-4 was preincubated (1 h at 37°C) with increasing concentrations of anti-IL-4 antiserum. The observed effect on CFU-M colony growth could be completely abrogated by this antiserum in a dose-dependent fashion (Fig. 3).

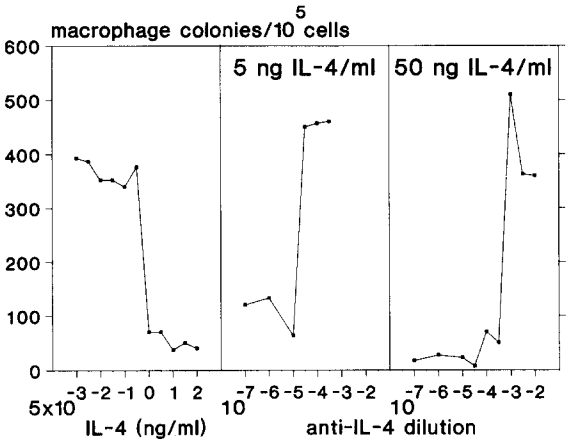

FIGURE 3. Effect of anti-IL-4 antiserum on inhibition of CFU-M colony formation by IL-4. Monocyte- and T cell-depleted bone marrow cells were cultured with GM-CSF (50 U/ml). The number of CFU-M colonies/10^5 cells is shown. Control CFU-M growth (culture without IL-4 added) was 513/10^5 cells.

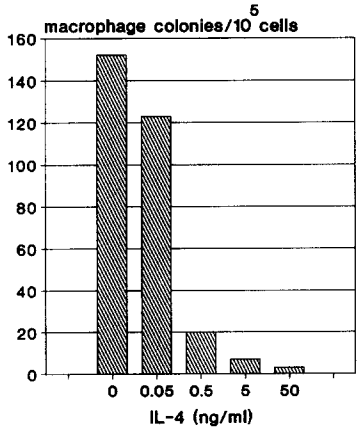

FIGURE 4. Influence of IL-4 on macrophage colony formation by monocyte- and T cell-depleted bone marrow cells, induced with EPO (1 U/ml) and M-CSF (1,000 U/ml). The number of CFU-M colonies/10^5 cells is shown.

M-CSF-stimulated Colony Formation. To study whether the inhibition of macrophage colony formation by IL-4 was dependent on the growth factor used to induce colony growth, increasing doses of IL-4 were added to monocyte- and T cell-depleted bone marrow cultures in which colony growth was induced with EPO and M-CSF. These experiments yielded a similar dose-dependent inhibition of CFU-M growth as observed in GM-CSF-induced cultures (Fig. 4).

Discussion

In this article we studied the influence of IL-4 on colony formation by bone marrow-derived hematopoietic progenitor cells. We observed that colony formation by monocytic precursor cells (CFU-M) was inhibited in a dose-dependent fashion, whereas colony growth of other lineages was not affected. Concentrations of 0.5 ng IL-4/ml were able to reduce CFU-M growth and profound inhibition was obtained at concentrations higher than 5 ng IL-4/ml.

Inhibition was still seen when a second, nonglycosilated IL-4 preparation was used and could be abrogated by heat inactivation or incubation of the IL-4 with a neutralizing anti-IL-4-antiserum, confirming that this inhibition was due to IL-4 and not to some nonspecific toxic contamination.

The IL-4-induced inhibition of macrophage colony formation was not only seen in cultures of light density and cultures of monocyte- and T cell-depleted bone marrow cells but was still observed on bone marrow cell suspensions enriched (± 50-fold compared with light density bone marrow cells) for $CD34^+$ $HLA\text{-}DR^+$ progenitor cells (Fig. 2). These results suggest a direct effect of IL-4 on monocytic progenitor cells.

The effect of IL-4 on CFU-M growth was not restricted to GM-CSF-stimulated colony growth, but was also observed in M-CSF-stimulated bone marrow cultures. Since colony growth of other lineages was not affected, these findings indicate that inhibition was not caused by interference of IL-4 with the receptors for colony-stimulating factors. We speculate that IL-4, in addition to possible synergism with growth factors such as EPO and G-CSF (3–5), acts directly on hematopoietic progenitor cells by either committing them to nonmonocytic lineages or by blocking the proliferation of monocyte-committed progenitor cells. We favor the latter hypothesis, since inhibition of CFU-M was not accompanied by a compensatory increase in colony formation of other lineages of differentiation. This might well correlate with data from others, describing IL-4-induced differentiation of mononuclear phagocytes (8–11).

In conclusion, we describe an IL-4-induced, specific inhibition of macrophage colony formation by bone marrow cells that appears to be exerted directly on the bone marrow progenitor cells. In addition to the production of lineage-specific growth factors, these results imply a mechanism by which hematopoiesis might be regulated in a lineage-specific fashion. To what extent this represents a physiological function of IL-4 remains to be elucidated.

Summary

We investigated the effects of human rIL-4 on in vitro hematopoiesis. A profound inhibition of macrophage colony formation by IL-4 was observed, whereas colony growth of other lineages was not affected. Inhibition of macrophage colony growth was not restricted to GM-CSF-induced colony growth but was also present in cul-

tures stimulated with M-CSF. This inhibition was not only observed in cultures of light density bone marrow cells, but also in cultures of monocyte- and T lymphocyte-depleted bone marrow cells. Since a similar inhibition was observed in cultures of $CD34^+HLA\text{-}DR^+$-enriched bone marrow cells, a direct action of IL-4 on monocyte-committed progenitor cells is suggested.

We thank Dr. J. E. de Vries (Unicet) for providing the anti-IL-4 antiserum and performing the IL-4 ELISA. We thank Mr. M. van Giessen, Dr. P. Herman (Essex), and Dr. P. P. Trotta (Schering Corp.) for providing the rIL-4.

Received for publication 4 April 1989 and in revised form 22 May 1989.

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