

## **Interleukin 6 Is a Permissive Factor for Monocytic Colony Formation by Human Hematopoietic Progenitor Cells**

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### **Summary**

Since monocytes and macrophages that arise during the culture of bone marrow progenitor cells are potential sources of interleukin 6 (IL-6), we investigated whether auto- or paracrine production of this factor is involved in colony formation by normal hematopoietic progenitor cells. We added a polyclonal anti-IL-6 antiserum and a monoclonal anti-IL-6 antibody to cultures of monocyte- and T cell-depleted bone marrow cells. Colony formation was stimulated with granulocyte/monocyte-colony-stimulating factor (GM-CSF), monocyte-CSF, or IL-3. Addition of anti-IL-6 antibody resulted in decreased numbers of monocytic colonies to 40–50% of control values, whereas the numbers of granulocytic colonies were not altered. The inhibitory effect was preserved in cultures of CD34<sup>+</sup>-enriched bone marrow cells. As a second approach, we added a monoclonal antibody directed against the IL-6 receptor to cultures of monocyte- and T cell-depleted bone marrow cells. This antibody almost completely inhibited the growth of monocytic colonies, again without decreasing the number of granulocytic colonies. Finally, the importance of IL-6 in monocytopoiesis was demonstrated in serum-deprived bone marrow cultures: addition of exogenous IL-6 to cultures stimulated with GM-CSF resulted in increased numbers of monocytic colonies. Our results indicate that the permissive presence of IL-6 is required for optimal monocytic colony formation by bone marrow progenitor cells.

IL-6 is involved in the development of B and T lymphocytes and is one of the major cytokines that induce the acute phase response upon infection or injury (1). In addition, IL-6 has been attributed a role in myelopoiesis. Alone, IL-6 does not induce colony formation by bone marrow progenitor cells, but it enhances IL-3-stimulated proliferation of myeloid progenitor cells (2) and the formation of monocytic colonies at suboptimal concentrations of monocyte (M)-CSF (3). Furthermore, it stimulates thrombocytopoiesis, both in vitro and in vivo (4, 5).

Colony formation assays have proved to be invaluable in the study of factors that regulate hematopoiesis. To study possible direct effects of cytokines, suspensions of purified bone marrow progenitor cells may be used. However, auto- or paracrine production of cytokines by progenitor cells or their offspring may still influence colony formation. Since monocytic cells are potent producers of IL-6 (1, 6), and these cells originate in cultures of bone marrow progenitor cells, we investigated the role of auto- and paracrine production of IL-6 on the development of colonies using neutralizing antibodies against IL-6 and the IL-6R. Since monocytes rap-

idly lose viability in serum-free conditions (7), we also tested whether addition of IL-6 resulted in enhanced monocytic colony formation in serum-deprived cultures.

### **Materials and Methods**

**Cytokines and Antibodies.** Human rIL-6 was kindly provided by Dr. L. A. Aarden (CLB, Amsterdam, The Netherlands); human rGM-CSF was provided by Sandoz Pharma BV (Uden, The Netherlands); human rM-CSF was a gift from Dr. P. Ralph (Cetus Corp., Emeryville, CA); human rG-CSF was a gift from Dr. B. Altrock (Amgen, Thousand Oaks, CA); human rIL-3 was a gift from Dr. G. Wagemaker (Netherlands Organization for Applied Scientific Research, Delft, The Netherlands). Mouse anti-human IL-6 mAb (neutralizing 10 U/ml IL-6 at a dilution of 1:10<sup>-5</sup>) and a polyclonal rabbit anti-human IL-6 (neutralizing 10 U/ml IL-6 at a dilution of 3:10<sup>-4</sup>) were raised against purified rIL-6. Anti-IL-6R antibody, PM-1 (8), was kindly provided by Dr. T. Kishimoto (Osaka University, Japan). This antibody competes with IL-6 for binding to the IL-6R (10 µg/ml neutralizes the activity of 2 ng/ml IL-6).

**Cell Preparations.** Bone marrow was obtained after informed consent from donors for allogeneic bone marrow transplantation

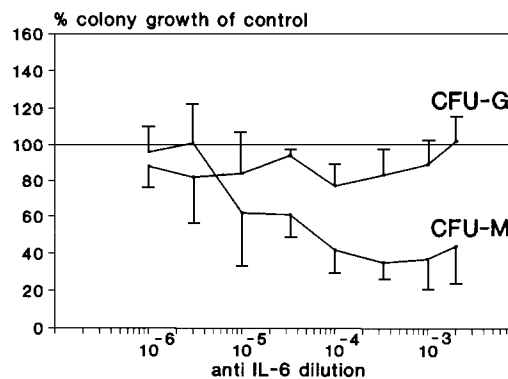
or from hernia nuclei pulposi patients who underwent laminectomy. Light density cells were isolated by Ficoll separation (1,000 g, 20 min,  $d = 1.077 \text{ g/cm}^3$ ). Interphase cells were depleted of monocytes by carbonyl iron phagocytosis (9), and T cells were removed by rosetting with sheep erythrocytes and Ficoll separation (10), yielding cell suspensions with <1% monocytes (staining with  $\alpha$ -naphthyl acetate esterase [ANAE]) and <1% T cells (staining with anti-CD3 [Becton Dickinson & Co., Mountain View, CA] and FITC-conjugated goat anti-mouse serum [GAM-FITC; Central Laboratory of the Red Cross Blood Transfusion Service, Amsterdam, The Netherlands]). From these suspensions, CD34<sup>+</sup>/HLA-DR<sup>+</sup> cells were isolated by FACS<sup>®</sup> (Becton Dickinson & Co.). Cells were preincubated with 5% (vol/vol) rabbit serum (30 min at 37°C) to prevent nonspecific Fc binding and stained with anti-HLA-DR (Becton Dickinson & Co.) and GAM-FITC (CLB). After incubation with murine serum (50%; vol/vol), blocking nonspecific binding of antibodies to the GAM-FITC, cells were stained with PE-conjugated anti-CD34 (MY10; Becton Dickinson & Co.).

**Bone Marrow Cultures.** Monocyte- and T cell-depleted bone marrow cells ( $0.3$  or  $0.5 \times 10^5/\text{ml}$ ) or CD34<sup>+</sup>/HLA-DR<sup>+</sup>-enriched bone marrow cells ( $10^4/\text{ml}$ ) were cultured in IMDM containing 20% FCS (Sanbio, Uden, The Netherlands),  $5 \times 10^{-5} \text{ M}$  2-ME (Sigma Chemical Co., St. Louis, MO), 0.5 g/liter deionized BSA, 0.47 g/liter human transferrin (Behringwerke, Hoechst, Amsterdam, The Netherlands),  $10^{-5} \text{ M}$  FeCl<sub>3</sub>·6H<sub>2</sub>O, and 0.98% methylcellulose. In serum-deprived cultures, the FCS was replaced by  $2 \times 10^{-4} \text{ M}$  cholesterol (Sigma Chemical Co.) and 10  $\mu\text{g}/\text{ml}$  insulin (Sigma Chemical Co.) as described (11). Colony formation was induced with G-CSF (10 ng/ml), M-CSF (1,000 U/ml), IL-3 (50 ng/ml), or GM-CSF (50 U/ml). Six replicates of 100  $\mu\text{l}$  were plated in flat-bottomed microtiter plates (Greiner, Alphen a/d Rijn, The Netherlands), except for experiments with CD34<sup>+</sup> cells, where two replicates of 1 ml were plated in 25-mm petri dishes (Greiner). Cultures were kept at 37°C in a fully humidified, 5% CO<sub>2</sub> atmosphere. CFU-G (>50 granulocytes) and CFU-M (>20 macrophages) were scored on day 14. Colonies were mass harvested or picked individually with a micropipette, and stained with ANAE or May-Grünwald Giemsa.

## Results

**Effect of Anti-IL-6 Antibody on Monocytic Colony Formation.** We investigated the role of endogenously produced IL-6 in serum-containing cultures of monocyte- and T cell-depleted bone marrow cells. Increasing concentrations of an anti-IL-6 mAb were added to cultures that were stimulated with GM-CSF (Fig. 1). A dose-dependent reduction of the number of monocytic colonies to 40% of control values was observed, with the number of granulocytic colonies unaffected. A similar effect was observed when a polyclonal anti-IL-6 antiserum was used. In the presence of this antiserum, CFU-G growth was  $110 \pm 27\%$  and CFU-M growth was  $50 \pm 11\%$  of control growth (mean  $\pm$  SD,  $n = 5$ ).

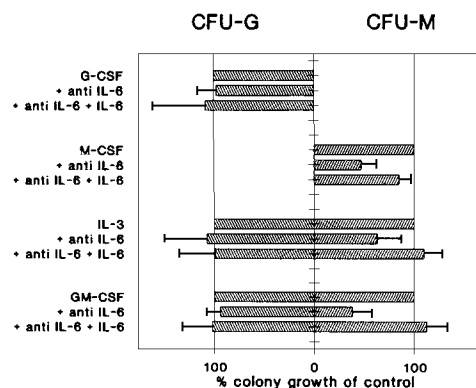
To test whether the specific inhibition of monocytic colony formation was dependent on the growth factor used to induce colony formation, we added anti-IL-6 mAb to cultures that were stimulated with G-CSF, M-CSF, or IL-3 (Fig. 2). In these studies, a similar inhibition of monocytic colony formation was observed, with the number of granulocytic colonies again unaffected.



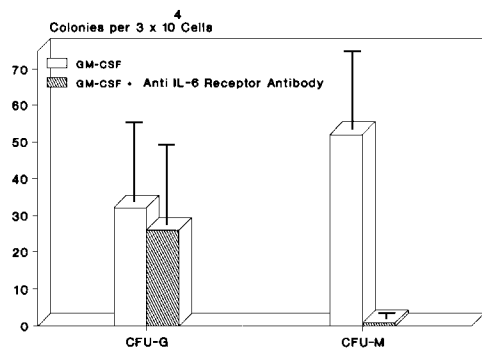
**Figure 1.** Effect of anti-IL-6 mAb on the formation of granulocytic and monocytic colony formation by monocyte- and T cell-depleted bone marrow cells. Data are expressed as the percentage of colony formation in control cultures without antibody added (mean  $\pm$  SD,  $n = 5$ ). Mean control growth was  $110 \pm 15$  CFU-G and  $87 \pm 16$  CFU-M/ $0.5 \times 10^5$  cells.

Inhibition of monocytic colony formation was not due to nonspecific toxicity of the antibody, since the numbers of granulocytic colonies were not affected, and the effect could be reversed by the addition of excess concentrations of exogenous IL-6 (200 ng/ml) (Fig. 2). Dose-dependent suppression of monocytic colony formation was preserved to the same degree in cultures of CD34<sup>+</sup> bone marrow cells stimulated with GM-CSF (50 U/ml). Anti-IL-6 mAb at a dilution of 1:10<sup>5</sup> to 1:200 suppressed CFU-M growth to  $56 \pm 5\%$  ( $n = 5$ , duplicate cultures) of control values, whereas the number of granulocytic colonies was not affected. Mean control growth was 17 CFU-G and 36 CFU-M/ $10^3$  cells.

**Effect of Anti-IL-6R Antibody on Monocytic Colony Formation.** To further substantiate the role of IL-6 in monocytic colony formation, we tested whether blocking of the IL-6R by an anti-IL-6R antibody was able to inhibit monocytic colony formation. Almost complete inhibition (to 2% of control values,  $n = 4$ ) of monocytic colony formation was observed in cultures containing the anti-IL-6R antibody. The number

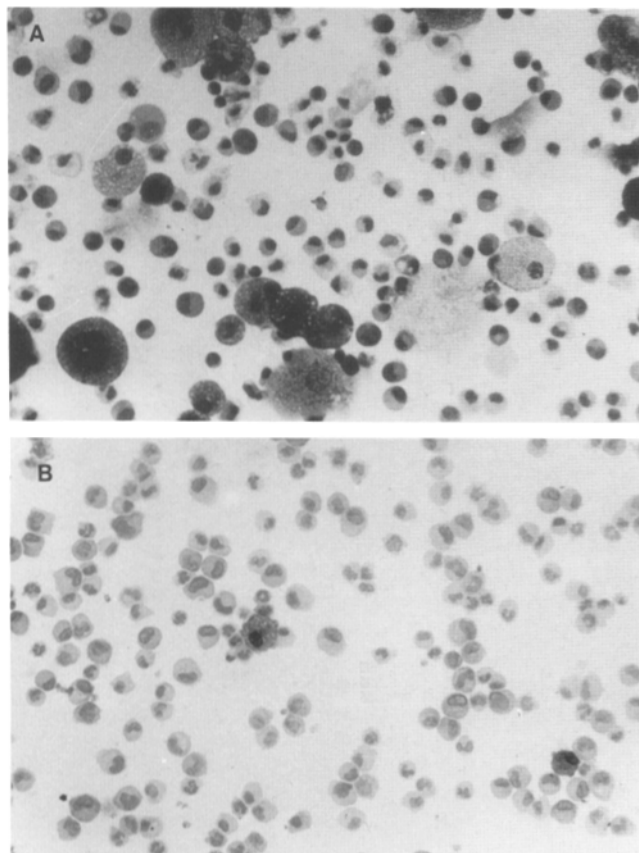


**Figure 2.** Effect of anti-IL-6 antibody ( $1:10^3$ ) on granulocytic and monocytic colony formation by monocyte- and T cell-depleted bone marrow cells stimulated with G-CSF (10 ng/ml), M-CSF (1,000 U/ml), IL-3 (50 ng/ml), or GM-CSF (50 U/ml). Data are expressed as the percentage of colony formation in control cultures without anti-IL-6 antibody (mean  $\pm$  SD,  $n = 5$ ).



**Figure 3.** Effect of anti-IL-6R antibody (10  $\mu\text{g/ml}$ ) on granulocytic and monocytic colony formation by monocyte- and T cell-depleted bone marrow cells stimulated with GM-CSF (50 U/ml). Mean values of four experiments  $\pm$  SD are given.

of granulocytic colonies was unaffected (Figs. 3 and 4), indicating that the effect was not due to toxicity of the antibody. Additionally, specificity could be confirmed since this antibody competes with IL-6 for binding to the IL-6R (8). In two cases tested, inhibition of monocytic colony formation to 2% and 3% of control values was reversed to 112% and



**Figure 4.** Photomicrograph of cell preparations prepared from cultures of monocyte- and T cell-depleted bone marrow cells cultured with (A) GM-CSF (50 U/ml) or (B) GM-CSF (50 U/ml) + anti-IL-6R antibody (1:10<sup>3</sup>). After 14 d of culture, cells were mass harvested and monocytic cells were stained with ANAE as described.  $\times 630$ .

**Table 1.** Effect of Interleukin 6 on Monocytic Colony Formation in Serum-deprived Cultures of Monocyte- and T Cell-depleted Bone Marrow Cells

Stimulus	Colonies/ $5 \times 10^4$ bone marrow cells			
	Exp. 1		Exp. 2	
	CFU-G	CFU-M	CFU-G	CFU-M
Culture medium	0	0	0	0
GM-CSF (50 U/ml)	27	23	77	3
GM-CSF (500 U/ml)	29	21	94	3
GM-CSF (50 U/ml) + IL-6 (200 ng/ml)	28	66	84	24
GM-CSF (500 U/ml) + IL-6 (200 ng/ml)	30	77	85	24

51% of control values by the addition of exogenous IL-6 (200 ng/ml).

**Effect of IL-6 on Monocytic Colony Formation in Serum-deprived Cultures.** Since monocytes rapidly lose viability in serum-free conditions, and serum may contain IL-6 or IL-6-inducing factors, we tested whether IL-6 was able to enhance monocytic colony formation in serum-deprived cultures of monocyte- and T cell-depleted bone marrow cells. In the presence of GM-CSF (50–500 U/ml), low numbers of monocytic colonies were obtained. In two experiments, addition of IL-6 resulted in a four- and eightfold increase in the number of monocytic colonies (Table 1). The number of granulocytic colonies was not affected.

## Discussion

In this report we show by three different approaches that IL-6 plays an important role in monocytic colony formation by bone marrow progenitor cells. Inhibition of monocytic colony formation by anti-IL-6R antibody was much more pronounced (98% inhibition) than inhibition by anti-IL-6 antibodies (50–60% inhibition). This difference might be explained by the existence of high and low affinity types of receptor-ligand binding between IL-6 and its receptor (12), the anti-IL-6 antibodies being able to compete only with the low affinity binding. Alternatively, the IL-6R may bind to more than one ligand, being the anti-IL-6R antibody able to block binding of both ligands.

These data underscore that in standard bone marrow cultures, on which the concept of regulation of hematopoiesis largely has been based, intermediate production of cytokines by the progenitor cells or their offspring may play an important role.

Several hypotheses may explain our data. IL-6 may act as a competence factor, inducing sensitivity of progenitor cells to growth factors, or as a progression factor, enabling cells

to proceed through the restriction point of the cell cycle. Alternatively, IL-6 may enhance progenitor cell survival by preventing apoptosis (programmed cell death). Recently, several authors suggested that hematopoietic growth factors prevent apoptosis in hematopoietic progenitor cells (13–15). Cells from IL-3, GM-CSF-, and G-CSF-dependent murine cell lines, died by apoptosis upon withdrawal of the relevant growth factor. Remarkably, IL-6 inhibited apoptosis that was induced by the expression of the putative tumor-suppressor protein p53 in a murine myeloid leukemia cell line (19). Serum starvation of human peripheral blood monocytes causes apop-

tosis, which can be prevented by the addition of IL-1 $\beta$ , TNF- $\alpha$ , or LPS (7). Although IL-6 did not prevent apoptosis in these experiments, prevention of apoptosis by IL-6 in more immature monocytic precursors cells, as studied in our experiments, remains possible.

If IL-6 has a similar permissive role in monocytopoiesis in vivo, this would add another level of control over the production of monocytes and macrophages. The high concentration of IL-6 in inflammatory tissues and the high titer in plasma upon infection and injury may be of relevance in this respect.

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