Effect of Interleukin-4 on the Growth of Granulocyte-Macrophage Progenitor Cells Stimulated by Hematopoietic Growth Factors

Tadatsugu SATO¹, Masahiro MISAGO¹, Jun-ichi TSUKADA¹, Makoto KIKUCHI,²
Susumu ODA¹, Shyozo CHIBA^{1,2} and Sumiya ETO¹

¹*First Department of Internal Medicine, School of Medicine, University of Occupational and Environmental Health, Japan. Kitakyushu 807, Japan*

²*Division of Hematology, School of Medical Technology, University of Occupational and Environmental Health, Japan. Kitakyushu 807, Japan*

Abstract : The effects of recombinant murine interleukin-4 (rmIL-4) on murine granulocyte-macrophage progenitor cells (CFU-GM) were investigated in the presence or absence of recombinant human granulocyte colony stimulating factor (rhG-CSF), recombinant murine granulocyte-macrophage colony stimulating factor (rmGM-CSF) or recombinant murine interleukin-3 (rmIL-3) both in serum-free and serum-containing cultures. IL-4 alone could not support any CFU-GM colony formation in both culture systems. In serum-free cultures, IL-4 inhibited CFU-GM colony formation stimulated by G-CSF, GM-CSF or IL-3 from whole bone marrow cells (whole BM cells), nonadherent, nonphagocytic and T cell-depleted BM cells (fractionated BM cells) or whole BM cells of 5-Fluorouracil (5-FU) treated mice. On the other hand, in serum-containing cultures, IL-4 inhibited CFU-GM colony formation stimulated by GM-CSF or IL-3, but IL-4 dose-dependently enhanced G-CSF-stimulated colony formation from both whole and fractionated BM cells. Morphological examinations revealed that IL-4 predominantly inhibited granulocyte-lineage colony formation stimulated by GM-CSF or IL-3 in both culture systems. When combined with G-CSF, IL-4 predominantly inhibited granulocyte-lineage colony formation and relatively increased macrophage-lineage colonies, but in serum-containing culture, IL-4 significantly increased macrophage-lineage colony formation. The results of the present study suggest that IL-4 has an inhibitory effect on the factors (G-CSF, GM-CSF, IL-3)-dependent CFU-GM colony formation in serum-free condition and enhances only G-CSF-stimulated CFU-GM colony formation in serum-containing condition.

Key words : IL-4, hematopoietic growth factors, CFU-GM, serum-free culture.

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Introduction

Differentiation and proliferation of hematopoietic progenitor cells require specific glycoproteins which are called colony stimulating factors (CSFs) (Clark & Kamen, 1987). Recently, four types of murine or human CSFs are highly purified and recombinant products are prepared (Cannistra & Griffin, 1988) — interleukin-3 (IL-3 or multi-CSF), granulocyte colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor

(GM-CSF) and macrophage colony stimulating factor (M-CSF).

On the other hand, other cytokines such as interleukin-1 (IL-1), interleukin-4 (IL-4) and interleukin-6 (IL-6), which were originally defined by their functional activities, have been found to have enhancing activities on the differentiation and proliferation of myeloid cells (Cannistra & Griffin, 1988; Metcalf, 1989).

IL-4/B-cell stimulatory factor-1 (BSF-1) was originally identified as a co-stimulator of the DNA synthesis in resting B-cells incubated with anti-IgM antibodies (Paul & Ohara, 1987). Besides the actions on B cells, IL-4 has now been shown to stimulate the growth of T cells, to act as a co-stimulator for the proliferation of mast cells, and to affect the proliferation of hematopoietic stem cells. Peschel *et al.* (1987) showed that IL-4 alone did not support any colony formation, but the addition of 30 U/ml of IL-4 to G-CSF led to an increase in size and number (2 to 3 times) of granulocyte-colonies (G-colonies). Rennick *et al.* (1987) observed that IL-4 enhanced the CFU-GM (granulocyte-macrophage colony forming unit in culture) colony formation stimulated by G-CSF and M-CSF, but inhibited the IL-3-dependent colony formation of granulocyte-macrophage progenitor cells (CFU-GM). Broxmeyer *et al.* (1988) reported that IL-4 (more than 100 U/ml) synergized with G-CSF, but not with GM-CSF, IL-3 or M-CSF to enhance the CFU-GM colony formation. In contrast, more recently, Kishi *et al.* (1989) showed that IL-4 alone could support GM-colonies, and that IL-4 was a multi-CSF similar to IL-3 and directly supported the proliferation of primitive hematopoietic progenitors. All these studies were carried out using culture medium containing serum.

Thus, it still remains unknown whether IL-4 itself supports the CFU-GM colony formation. Furthermore, because 10 to 30% of fetal calf serum (FCS) was used in culture systems of these reports, the possibility also still remains that FCS affects the CFU-GM colony formation. In this paper, we report the effects of recombinant murine IL-4 (rmIL-4) on the colony formation from murine BM cells in both serum-free and -containing agar culture systems. This analysis was carried out in the absence or presence of G-CSF, GM-CSF or IL-3. Our results indicate that IL-4, when without serum, has an inhibitory effect on the factor-dependent colony formation of CFU-GM in serum-free condition, and has a synergistic effect with G-CSF in serum-containing condition.

Materials and Methods

Mice

Eight to 12 weeks old female BALB/c mice (Kyudo Co. Ltd., Tosu, Saga, Japan) were used in all experiments.

Cell preparation

Bone marrow (BM) nonadherent cells were obtained by culturing in plastic flasks. 1×10^6 cells/ml bone marrow cells suspended in Iscove's modified Dulbecco's tissue culture

medium (IMDM; GIBCO Laboratories, Grand Island, NY) containing 10 mg/ml deionized Cohn fraction V BSA (Armour Pharmaceutical Co., Kankakee, IL) were incubated twice in plastic culture flasks (Falcon 3024; Becton Dickinson and Co., Oxnard, CA) for 60 min at 37°C. Nonadherent cells obtained by rinsing were further incubated with 10% KAC-2; a silica suspension (Otsuka Bioassay Laboratories, Tokyo, Japan) for 60 min at 37°C to deplete phagocytic cells. After incubation, nonadherent and nonphagocytic cells were separated by density centrifugation using Lympholyte-M cell separation medium (Cedarlane Laboratories, Ontario, Canada), and washed twice with 10 mg/ml BSA containing IMDM. T-cell depletion was performed by the treatment with monoclonal antibody and complement. To the pellet of 1×10^7 nonadherent and nonphagocytic cells prepared as described above, 1:500 diluted anti-Thy 1.2 monoclonal antibody (Cedarlane Laboratories) was added, and then the cells were incubated for 60 min at 4°C. After the addition of low-Tox-M Rabbit Complement (1:10, Cedarlane Laboratories), the cells were further incubated for 60 min at 37°C. And then the viable cells were separated by Lympholyte-M cell separation medium and washed twice. These obtained cells were used as fractionated BM cells (nonadherent, nonphagocytic and T cell-depleted BM cells). The recovery of fractionated BM cells was usually less than 5% of the original BM cells and almost all these cells were viable as detected by a trypan-blue dye exclusion test. Routinely, the contamination of α -Naphthyl Butyrate esterase staining positive cells in the final cell suspensions was less than 1%.

BM cells of 5-FU treated mice

5-fluorouracil (5-FU; Kyowa Hakko Kogyo Co. Ltd., Tokyo, Japan) was administered intravenously through the tail vein of mice at 150 mg/kg. BM cells were harvested 4 days after 5-FU injection. The progenitor cells in these BM cells were highly enriched for the dormant primitive hematopoietic progenitors (Hodgson & Bradley, 1979; Stanley *et al.*, 1986).

Reagents

RmIL-4 was kindly provided by Ono Pharmaceutical Co. (Osaka, Japan). It had a specific activity of 1×10^8 U/mg protein by an anti-IgM costimulation assay (Severinson *et al.*, 1987). RmIL-3 was purchased from Genzyme Corporation (Boston, MA). It had a specific activity of 10^7 U/mg by proliferation assay using IL-3 dependent FDC-P2 cells (Prestidge *et al.*, 1984). RhG-CSF was generously provided by Chugai Pharmaceutical Co. (Tokyo, Japan), and had a specific activity of 2×10^8 U/mg (Nagata *et al.*, 1986; Tsuchiya *et al.*, 1987). RmGM-CSF was also provided by Sumitomo Pharmaceutical Co. (Osaka, Japan). It had a specific activity of 5×10^8 U/mg. One unit of activity is defined as the amount required to stimulate formation of one CFU-GM colony in soft agar cultures with 7.5×10^4 murine whole BM cells (Prestidge *et al.*, 1984). RhG-CSF has no species specificity, and so, it is fully available for studying in culture systems for murine cells (Zsebo *et al.*,

1986).

Each factor was plated alone in whole BM cultures over various dose ranges (rmGM-CSF, 0–100 ng/ml; rhG-CSF, 0–100 ng/ml; rmIL-3, 0–400 U/ml) and a plateau dose of each factor was determined from dose-responsive curves of total colony formation at 4 days of culture. These doses were used alone or in combination. The doses used were 0.1 ng/ml for rmGM-CSF, 20 ng/ml for rhG-CSF or 200 U/ml for rmIL-3, respectively.

CFU-GM colony assay

Serum-free culture

A serum-free culture system was performed using the method of Stewart *et al.* (1984) with some modifications (Sato *et al.*, 1988). 1×10^5 cells/ml of whole BM cells of normal or 5-FU treated mice, or 4×10^4 cells/ml of fractionated BM cells, were cultured in 35×10 mm plastic Petri dishes (Falcon 3001) containing IMDM with 0.3% agar (Difco Laboratories, Detroit, MI), 10 mg/ml deionized Cohn fraction V BSA, 388 μ g/ml human transferrin (Sigma Chemical Co., St. Louis, MO) saturated with FeCl_3 , 7.8 μ g/ml cholesterol (Wako Pure Chemical Industries, Osaka, Japan), 7.5×10^{-5} M 2-mercaptoethanol (2ME; Wako) and various concentrations of IL-4 with or without IL-3, G-CSF or GM-CSF. Cells were cultured for 7 days at 37°C in a humidified atmosphere of 5% CO_2 . When BM cells of 5-FU treated mice were used, cells were cultured for 12 days under the same conditions as described above.

A CFU-GM colony was defined as an aggregate consisting of more than 40 cells. If an aggregate consisted of 10 to 40 cells, it was scored as a cluster. *In situ* esterase-double staining was performed by the techniques previously described for identifying the colony types (Tsukada *et al.*, 1987). Briefly, Whatman #1 filter paper disks were placed over the agar layer in the Petri dish, and the agar was dried by blowing cold air over it until the filter paper was dry. The fine agar film was stained by using esterase staining kits (Muto Chemical Co. Ltd., Tokyo, Japan).

We previously reported that in these serum-free cultures, CFU-GM colony formation from whole BM cells was supported at the same degree in serum-containing (FCS 20%) cultures when stimulated by serum-free pokeweed mitogen-stimulated spleen cell conditioned medium (PWM-SCM) (Sato *et al.*, 1988).

Serum-containing culture

1×10^5 BM cells were cultured in Petri dishes containing IMDM with 0.3% agar, 20% FCS (Boehringer Mannheim GmbH, West Germany), 6×10^{-5} M 2ME and the reagents described above. After incubation under the same conditions as those in serum-free cultures, colonies were counted and stained. In some experiments, the concentration of FCS was increased up to 40%.

Statistics

All experiments were repeated at least three times, and the representative results are given here. Results are expressed as Mean $\pm$ SE of triplicate cultures. The Student's *t* test was used to determine the statistical significance.

Results

Effect of IL-4 on CFU-GM colony formation from whole BM cells in serum-containing cultures

Whole murine BM cells were cultured for 7 days at FCS concentrations of either 20% or 40% with various doses of IL-4. Each concentration of IL-4 up to 500 U/ml could support only a few clusters but not a colony at FCS concentrations up to 40% (data not shown). These observations indicate that IL-4 itself does not have any CFU-GM colony supporting ability even in the presence of serum.

Effect of IL-4 on CFU-GM colony formation from whole BM cells with or without respective G-CSF and GM-CSF in serum-free cultures

IL-4 could not support any CFU-GM colonies in serum-free cultures (Fig. 1), as it did in serum-containing cultures. Therefore, various doses of IL-4 were added to serum-free cultures of a CFU-GM colony stimulated by an optimal dose of either G-CSF or GM-CSF to elucidate the synergistic effect of IL-4 (Fig. 1). IL-4 inhibited CFU-GM colony forma-

Fig. 1. Effect of IL-4 on CFU-GM colony formation from whole BM cells in serum-free cultures. CFU-GM colony formation stimulated by IL-4 alone (●), IL-4 with 20 ng/ml of G-CSF (○) or 0.1 ng/ml of GM-CSF (△) were studied. Each point represents Mean $\pm$ SE of triplicate cultures. * = $P < 0.05$, ** = $P < 0.01$ when compared with control values (IL-4 not added).

tion stimulated by G-CSF dose-dependently, and also inhibited CFU-GM colony formation by GM-CSF. The maximum inhibitory effect of IL-4 on G-CSF- or GM-CSF-dependent CFU-GM colony formation was observed at more than 20 U/ml of IL-4, respectively.

Effect of IL-4 on factor (G-CSF, GM-CSF or IL-3)-dependent CFU-GM colony formation from fractionated BM cells in serum-free and serum-containing cultures

To elucidate whether the inhibitory effect of IL-4 on CFU-GM colony formation was direct action or indirect action through the accessory cells, nonadherent, nonphagocytic and T cell-depleted BM cells (fractionated BM cells) were used as target cells against IL-4, and the results in serum-free cultures were compared with those in serum-containing cultures (Table 1). The addition of 50 U/ml of IL-4 to the optimal concentrations of G-CSF, GM-CSF or IL-3 inhibited CFU-GM colony formation from fractionated BM cells in serum-free cultures. In serum-containing cultures, IL-4 also inhibited GM-CSF- or IL-3-dependent CFU-GM colony formation. However, IL-4 enhanced CFU-GM colony formation stimulated by G-CSF by 2 to 3 fold. This enhancing effect was also observed in CFU-GM colony formation from whole BM cells in serum-containing cultures (data not shown). Morphological examinations revealed that IL-4 inhibited mainly granulocyte-lineage colony

Table 1. CFU-GM colony formation from fractionated bone marrow cells and types of colonies formed in serum-free and -containing (FCS 20%) cultures

		Total colony number (/4×10 ⁴ cells)	Types of colonies		
			G	G/M	M
		serum-free cultures			
G-CSF		71±3.6	21	36	14
G-CSF	+ IL-4	46±6.2 [*]	1	14	31
GM-CSF		221±22.4	0	73	148
GM-CSF	+ IL-4	150±5.9 [*]	0	20	131
IL-3		247±6.1	20	82	146
IL-3	+ IL-4	37±4.4 [*]	2	7	28
		serum-containing cultures			
G-CSF		60±4.4	18	36	6
G-CSF	+ IL-4	148±4.7 [*]	0	44	104
GM-CSF		400±6.2	20	160	220
GM-CSF	+ IL-4	280±6.4 [*]	14	56	210
IL-3		418±9.3	20	230	168
IL-3	+ IL-4	202±3.4 [*]	0	40	162

Additional effects of IL-4 to G-CSF, GM-CSF and IL-3 were investigated for CFU-GM colony forming ability from fractionated bone marrow cells. The concentration of each growth factor was 20 ng/ml for G-CSF, 0.1 ng/ml for GM-CSF and 200 U/ml for IL-3, respectively, in which the numbers of CFU-GM colonies formed from whole bone marrow cells reached at plateau level. IL-4 was used at the concentration of 50 U/ml. Values are represented as Mean $\pm$ SE of triplicate cultures. * = $P < 0.01$ when compared with control values (IL-4 not added).

formation, but not macrophage-lineage colony formation and that the ratio of macrophage-lineage colonies was relatively increased when BM cells were stimulated by IL-3 or GM-CSF in combination with IL-4. However, when combined with G-CSF, IL-4 absolutely increased macrophage-lineage colony formation in serum-containing cultures. In serum-free cultures, IL-4 combined with G-CSF inhibited granulocyte-lineage colony formation, as it did when combined with IL-3 or GM-CSF. These results strongly suggest that the inhibitory effect of IL-4 on factor-dependent CFU-GM colony formation from BM cells is not affected by adherent cells, phagocytic cells and T cells, and that IL-4 acts as an enhancing factor on G-CSF-dependent CFU-GM colony formation under the presence of serum.

Effect of IL-4 on factor (G-CSF, GM-CSF or IL-3)-dependent CFU-GM colony formation from BM cells of 5-FU-treated mice in serum-free cultures

It was reported that Thy-1 is an antigen found not only on mature and immature T cells, but also on mouse hematopoietic stem cells including CFU-GM (Miller *et al.*, 1985). Therefore, the procedure of anti-Thy1.2 antibody and complement treatment may cause the depletion of some special populations of CFU-GM. To obtain the whole population of

Fig. 2. Effect of IL-4 on CFU-GM colony formation from BM cells treated with 5-FU 4 days previously in serum-free cultures. Various factors alone and in combination with 50 U/ml of IL-4 were studied. The doses used were 20 ng/ml for G-CSF, 0.1 ng/ml for GM-CSF or 200 U/ml for IL-3, respectively. Each point represents Mean $\pm$ SE of triplicate cultures scored on day 12 of culture. * = $P < 0.01$ when compared with control values (IL-4 not added).

Fig. 3. Effect of IL-4 on CFU-GM colony formation from fractionated BM cells. BM cells were stimulated by various doses of IL-4 with 20 ng/ml of G-CSF both in serum-free (●) cultures and in serum-containing (○) cultures (FCS 20%). Each point represents Mean $\pm$ SE of triplicate cultures. * = $P < 0.05$, ** = $P < 0.01$ when compared with control values (IL-4 not added).

CFU-GM, BM cells were prepared from mice injected with 5-FU 4 days previously. Figure 2 shows that IL-4 inhibits CFU-GM colony formation stimulated by either G-CSF, GM-CSF or IL-3 from 5-FU-treated BM cells in serum-free cultures. This indicates that IL-4 truly inhibits the factor-dependent CFU-GM colony formation in the serum-free culture system.

Dose-responsive curves of addition of IL-4 to G-CSF-stimulated CFU-GM colony formation from fractionated BM cells in serum-free and -containing cultures

IL-4 had different effects on CFU-GM colony formation in serum-free cultures and in serum-containing cultures when combined with G-CSF, as shown in Table 1. Therefore, the dose-responsive effect of added IL-4 to G-CSF stimulated-CFU-GM colony formation from fractionated BM cells in serum-free or -containing cultures was studied. IL-4 syner-

gistically stimulated CFU-GM colony formation with G-CSF in serum-containing cultures by 2 to 3 fold, but inhibited dose-responsively G-CSF-dependent CFU-GM colony formation in serum-free cultures (Fig. 3).

These results indicate that IL-4 requires the presence of serum to synergize with G-CSF.

Discussion

In this study, it was shown that IL-4 alone was incapable of stimulating CFU-GM colony formation, and inhibited the factors-dependent CFU-GM colony formation, especially granulocyte-lineage colony formation, in serum-free cultures. Several investigators also reported that IL-4 alone was unable to form CFU-GM colonies (Peschel *et al.*, 1987; Rennick *et al.*, 1987; Broxmeyer *et al.*, 1988). But Kishi *et al.* (1989) recently reported that IL-4 itself could support GM-colony formation at the concentrations of more than 100 U/ml from whole murine bone marrow cells in 30% FCS contained cultures. Their results are in direct conflict with the previous results and ours. They considered that the differences of their results might be due to the FCS concentration in the culture system, because they studied under the condition of FCS 30%, which was higher than that of previous reports. In the present study, we carried out the experiments using serum-containing cultures at FCS concentrations up to 40%, and showed that IL-4 could not support any CFU-GM colony formation even at the concentration of 500 U/ml. The reason for the differences between their results and ours is still unclear. It may be due to the difference of the batches of FCS because serum is considered to contain various identified and/or unidentified factors which affect the colony forming ability (Dainiak, 1985). Therefore, special batches of FCS, which are able to support large numbers of colonies, are selected for studying *in vitro* colony formation (Metcalf, 1984).

Our results indicate that IL-4 dose-dependently inhibits the factor-dependent CFU-GM colony formation stimulated by IL-3, GM-CSF and G-CSF in serum-free cultures, and that those inhibitory effects are also observed in CFU-GM colony formation from nonadherent, nonphagocytic and T cell-depleted BM cells and from BM cells of 5-FU treated mice. Previous reports indicated that IL-4 augmented CFU-GM colony formation when combined with G-CSF (Peschel *et al.*, 1987; Rennick *et al.*, 1987; Broxmeyer *et al.*, 1988). In our study in serum-containing cultures, we also showed that IL-4 combined with G-CSF stimulated synergistically CFU-GM colony formation. However, in serum-free cultures, IL-4 inhibited G-CSF-dependent CFU-GM colony formation dose-dependently. It is still unclear why the effects of IL-4 on G-CSF-dependent CFU-GM colony formation were different in serum-containing and -free cultures. In serum-containing cultures, the involvement of unidentified serum-borne factors in CFU-GM colony formation cannot be excluded, and so, it appears that some factors in the serum may act with IL-4 synergistically. It may be also concluded that hematopoietic factors are stage-specific, and so, the combination of

several hematopoietic growth factors may be required for the optimal colony formation in serum-free cultures (Kishi *et al.*, 1989; Sonoda *et al.*, 1988).

As for the indirect mechanism of the inhibitory effect of IL-4, Peschel *et al.* (1989) showed that IL-4 induced inhibitory activity in BM-derived stromal cell monolayers, and they supposed that macrophages in stromal layers may release an inhibitory substance in the presence of IL-4. Indeed, IL-4 has direct influences on macrophages (Crawford *et al.*, 1987; Zlotnik *et al.*, 1987) and our results also indicate that IL-4 relatively preserve macrophage-lineage colonies. But, recently, it is indicated that IL-4-activated monocytes from peripheral blood can secrete G-CSF and M-CSF (Wieser *et al.*, 1989) and that IL-4 inhibits the production of tumor necrosis factor- α , which inhibits CFU-GM colony formation stimulated by hematopoietic growth factors (Broxmeyer & Williams, 1988), from lipopolysaccharide stimulated monocytes (Hart *et al.*, 1989). Therefore, more detailed studies are required to clarify the role of macrophages in the IL-4 mediated inhibitory effect on progenitor cells *in vitro*.

We conclude that IL-4 has unique bipotential effects, mainly inhibitory and synergistic in some aspects, on CFU-GM. Thus, from our results that IL-4 inhibits the factor-dependent CFU-GM colony formation with either G-CSF, GM-CSF or IL-3 in serum-free cultures, it can be stated that IL-4 is added as a new factor to the previously described myelo-suppressive factors, such as prostaglandins, lactoferrin, interferons and tumor necrosis factor (Broxmeyer & Williams, 1988). Although further studies are required to clarify the role of IL-4 in granulopoiesis, the results reported by us and others indicate that IL-4 is an important regulator of hematopoiesis.

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インターロイキン 4 の造血因子刺激顆粒球・マクロファージ系前駆細胞増殖に対する効果

佐藤 忠嗣¹・三砂 将裕¹・塚田 順一¹・菊池 亮¹
 織田 進¹・千葉 省三^{1,2}・江藤 澄哉¹

¹産業医科大学第一内科学教室 ²産業医科大学医療技術短期大学血液学

要 旨: 遺伝子組換え型インターロイキン 4 (IL-4) のマウス顆粒球・マクロファージ系前駆細胞 (CFU-GM) 増殖に対する効果について、無血清および血清培養法を用いて検討した。また、G-CSF, GM-CSF あるいは IL-3 による CFU-GM 形成に対する IL-4 の効果についても検討した。1) 両培養系において、IL-4 単独では CFU-GM コロニーを形成しなかった。2) IL-4 は、無血清培養系において、全骨髓細胞、非付着非貧食非 T 骨髓細胞あるいは 5-Fluorouracil 処理骨髓細胞からの、G-CSF, GM-CSF, IL-3 による CFU-GM コロニー形成を抑制した。3) IL-4 は、血清培養系では、非付着非貧食非 T 骨髓細胞を用いた場合、GM-CSF および IL-3 による CFU-GM 形成を抑制したが、G-CSF による CFU-GM 形成を濃度依存性に増加させた。4) 両培養系において、IL-4 は、特に、GM-CSF, IL-3 による顆粒球系コロニー形成を抑制した。G-CSF を同時添加した場合、IL-4 は、無血清培養系では顆粒球系コロニー形成を抑制し、マクロファージ系コロニー形成を相対的に増加させたが、血清培養系では、マクロファージ系コロニー形成を著明に増加させた。以上の成績から、IL-4 は、血清の非存在下では、造血因子刺激 CFU-GM 増殖に対して抑制的に働くが、血清の存在下では G-CSF 刺激 CFU-GM 形成を増加させる作用があることが明らかとなった。

J. UOEH (産業医大誌), 12 (2): 163-174 (1990)