Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 765–769, February 1991
Cell Biology

Direct and synergistic effects of interleukin 11 on murine hemopoiesis in culture

(hemopoietic stem cell/growth factor/interleukin)

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Communicated by Helen M. Ranney, October 26, 1990 (received for review August 6, 1990)

ABSTRACT **We have examined the effects of a stromal cell-derived cytokine designated interleukin 11 (IL-11) on the proliferation of murine hemopoietic progenitors in methylcellulose culture. COS cell-conditioned medium containing IL-11 supported formation of granulocyte/macrophage colonies and a small number of multilineage colonies including blast cell colonies in cultures of marrow cells from normal mice. When tested with marrow cells harvested 2 days after injection of 5-fluorouracil at 150 mg/kg, IL-11 enhanced interleukin 3-dependent colony formation, whereas IL-11 alone supported only scant colony formation. Serial observations (mapping studies) of cultures of post-5-fluorouracil spleen cells indicated that the mechanism of the synergistic effect of IL-11 is to shorten the dormant period of stem cells, an effect very similar to that of interleukin 6. When pooled blast cells were plated into medium containing IL-11 and erythropoietin, only macrophage colonies were observed. Thus, IL-11 can directly support the proliferation of committed macrophage progenitors and, like interleukin 6 and granulocyte colony-stimulating factor, act synergistically with interleukin 3 to shorten the G_0 period of early progenitors.**

Hemopoietic proliferation appears to be supported by a cascade of growth factors, each directed at different developmental stages (1). In this model, proliferation and maturation of monopotent progenitors are controlled by lineage-specific growth factors such as erythropoietin (Ep), interleukin 5, and granulocyte colony-stimulating factor (G-CSF). Proliferation of more primitive, and thus multipotential, progenitors appears to be regulated by such factors as granulocyte/macrophage colony-stimulating factor, interleukin 3 (IL-3) and, in mice, interleukin 4.

In steady-state bone marrow, the hemopoietic stem cells thought to be in a quiescent state termed G_0 divide only infrequently to yield actively proliferating multipotential progenitors. The mechanisms regulating the cell division of dormant hemopoietic stem cells remained unknown until recently. Although IL-3 and interleukin 4 support proliferation of early multipotential progenitors, these factors do not trigger stem cells in G_0 into cell proliferation but rather support the proliferation of progenitors only after they exit from G_0 (2, 3). In our laboratory, we have developed a blast-cell colony assay that measures the proliferation of progenitors after they exit from the G_0 state (4, 5). By serial observation of blast-cell colony development (mapping method), we found that interleukin 6 (IL-6) (6) and G-CSF (7) can shorten the G_0 period of progenitors for the blast-cell colonies.

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Recently, another lymphohemopoietic cytokine, interleukin 11 (IL-11), was identified in medium conditioned by a cell line derived from primate bone marrow stromal cells (PU-34) (8). This cytokine was identified as a molecule mitogenic for the IL-6-dependent plasmacytoma T1165. Subsequently, we studied the effects of IL-11 in other IL-6-dependent culture systems. Here we report on the effects of IL-11 on the proliferation in culture of early murine progenitors, including blast-cell colony-forming cells. The results indicate that IL-11, similar to IL-6, is a synergistic factor for IL-3-dependent proliferation of primitive progenitors, and that part of the synergism is to shorten the G_0 period of the stem cells. In contrast to IL-6, however, IL-11 preferentially stimulates only macrophage proliferation in secondary cultures of pooled blast cells.

MATERIALS AND METHODS

Cell Preparation. Female BDF_1 mice, 10–15 weeks old, were obtained from ARS Sprague–Dawley (Indianapolis). Single-cell suspensions were prepared from pooled femurs or spleens of three mice. Light density (<1077) mononuclear cells were collected from the interface of Ficoll-Paque after centrifugation at 400 × *g*. After overnight adherence of these cells to plastic dishes, nonadherent mononuclear cells were harvested.

5-Fluorouracil (5-FU) (Adria Laboratories) was administered i.v. through the tail veins of mice at 150 mg/kg of body weight (5, 9). Bone marrow and spleen cells were harvested 2 and 4 days after 5-FU injection, respectively.

Factors. The source of IL-11 was conditioned medium (CM) from COS-1 cells transfected with cDNA encoding the murine plasmacytoma-stimulatory activity (8). The source of recombinant murine IL-3 was medium conditioned by Chinese hamster ovary (CHO) cells that had been genetically engineered to produce murine IL-3 to high titer (≈30,000 units per ml). Recombinant human IL-6 with specific activity of 4×10^6 units per mg of protein and purified recombinant human Ep with specific activity of 1.2×10^5 units per mg of protein were provided by R. Steinbrink and P. Schendel of the Genetics Institute (Cambridge, MA). Partially purified human urinary Ep with 370 units of activity per mg was provided by Makoto Kawakita (Kumamoto University Medical School, Kumamoto, Japan).

Clonal Cell Culture. Methylcellulose cell cultures were established in 35-mm Lux suspension culture dishes (5221R,

Abbreviations: CM, conditioned medium; Ep, erythropoietin; G-CSF, granulocyte colony-stimulating factor; GEMM, granulocyte/erythrocyte/macrophage/megakaryocyte; IL-3, interleukin 3; IL-6, interleukin 6; IL-11, interleukin 11; 5-FU, 5-fluorouracil.

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Table 1. Colony formation from marrow cells of normal mice

Factors	Colonies, no.						
	GM	Mast	M	GMM	GEMM	Bl	Total
Ep, mock CM (1:100)	184	0	0	0	0	0	184
Ep, IL-3 (100 units per ml)	452	64	2	6	9	0	533
Ep, IL-11 (1:100,000)	211	0	0	0	0	0	211
Ep, IL-11 (1:10,000)	208	0	0	0	0	0	208
Ep, IL-11 (1:1000)	270	0	0	0	0	0	270
Ep, IL-11 (1:100)	339	5	0	2	2	3	351

Data represent number of colonies from 8×10^4 cells. Concentration of urinary Ep was 2 units per ml. GM, granulocyte/macrophage colonies; Mast, mast cell colonies; M, megakaryocyte colonies; GMM, granulocyte/macrophage/megakaryocyte colonies; GEMM, granulocyte/erythrocyte/macrophage/megakaryocyte colonies; Bl, blast-cell colonies.

Nunc). One milliliter of culture contained 2×10^4 marrow cells from normal mice, 5×10^4 marrow cells or 1×10^6 spleen cells from 5-FU-treated mice, α-medium (Flow Laboratories), 1.2% (1500 centipoises) methylcellulose (Fisher), 30% fetal calf serum (HyClone), 1% deionized fraction V bovine serum albumin (Sigma), 1×10^{-4} M 2-mercaptoethanol (Eastman), and hemopoietic factors. Dishes were incubated at 37°C in a humidified atmosphere flushed with 5% CO_2. Except for megakaryocyte colonies, colonies consisting of 50 or more cells were scored on an inverted microscope on the specified day of incubation. Megakaryocyte colonies were scored when they contained four or more megakaryocytes.

Blast-Cell Colony Replating. The hemopoietic potential of the blast-cell colonies was determined by blast-cell colony replating. Between day 5 and 15 of incubation, individual blast-cell colonies containing 50–150 cells were picked with an Eppendorf pipet and replated in secondary methylcellulose cultures containing human urinary Ep at 2 units per ml and 1% (vol/vol) concentrated (×20) supernatant of cultures of WEHI-3 cells.

Blast cells were also used as pure target populations of hemopoietic cells to determine whether the observed effects of IL-11 were direct or due to the release of other factors. One million day 4 post-5-FU spleen cells were cultured in the presence of IL-3 at 100 units per ml. On day 8 of culture, individual blast-cell colonies (between 50 and 150 cells) were picked from cultures, pooled, washed twice with medium, and replated in secondary cultures each containing different combinations of factors.

RESULTS

Colony Formation from Marrow Cells of Normal Mice. First, we carried out dose–response studies with 1:100 to 1:100,000 dilutions of IL-11 using colony formation from marrow cells of normal mice. IL-11 gave rise to colonies in a dose-dependent manner, and a 1:100 dilution of IL-11 supported maximal colony formation. However, the total number of colonies detected on day 8 or day 16 of incubation

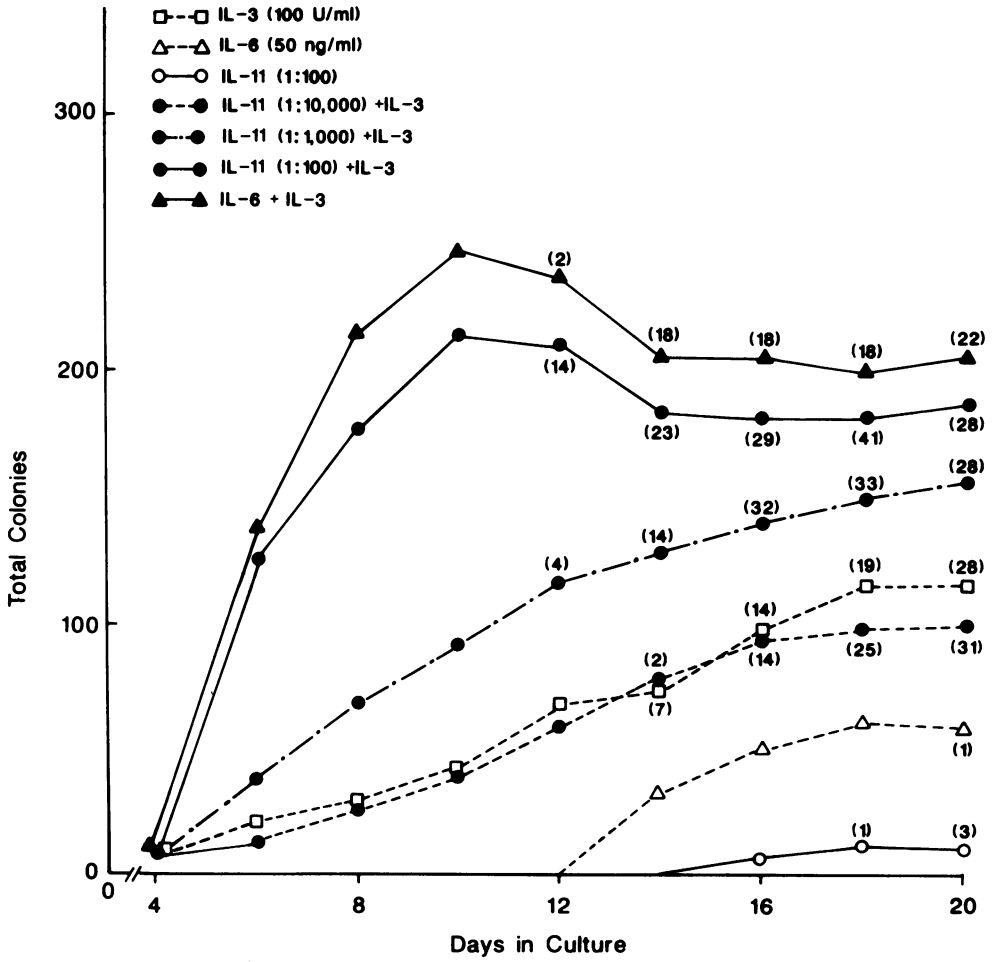

FIG. 1. Time course of colony formation from four plates each containing 5×10^4 marrow cells of 5-FU-treated mice, Ep at 2 units/ml, and designated factors. The numbers in parentheses indicate those of multilineage (GEMM and granulocyte/macrophage/megakaryocyte) colonies. There were no colonies in the plates containing IL-11 in 1:1000 dilution.

with IL-11 was significantly lower than in cultures with IL-3. The types of colonies identified on day 16 are shown in Table 1. The colonies found in IL-11-containing cultures were predominantly of the granulocyte/macrophage type, although some multilineage [granulocyte/macrophage/megakaryocyte (10) and granulocyte/erythrocyte/macrophage/megakaryocyte (GEMM) (10, 11)] colonies were also seen. IL-11 in a 1:100 dilution supported formation of three blast-cell colonies.

Colony Formation from Marrow Cells of 5-FU-Treated Mice. The observation that IL-11 supported formation of blast-cell colonies in cultures of marrow cells of normal mice is similar to our previous observation with IL-6 (6) and suggested the possibility that IL-11 might also act synergistically with IL-3 in supporting the proliferation of primitive progenitors. To test this possibility, we examined colony formation from marrow cells harvested 2 days after injection of 5-FU at 150 mg/kg (5, 9) in cultures established in the presence of IL-11, IL-6, IL-3 singly and in various combinations; the results are presented in Fig. 1. Addition of IL-11 at final dilutions of 1:100 and 1:1000 to an optimal concentration of IL-3 significantly enhanced colony formation. In particular, in the presence of a 1:100 dilution of IL-11 and IL-3 the kinetics of colony formation was accelerated as compared with that supported by the individual factors. The time course of colony formation as well as the total number of colonies supported were similar to those observed with the combination of IL-6 and IL-3. IL-11 alone in a 1:100 dilution supported scant colony formation after a long period of incubation. These results indicated that IL-11 enhances IL-3-dependent proliferation of primitive progenitors.

Serial Observations of Blast-Cell Colony Development from Day 4 Post-5-FU Spleen Cells. To further analyze the synergistic effect of IL-11, we serially plotted the growth rates of individual blast-cell colonies through culture mapping studies. For this purpose, we used spleen cells harvested 4 days after injection of 5-FU as we had reported (6, 7). One million day 4 post-5-FU spleen cells were plated per dish in the presence of Ep at 2 units per ml and either IL-3 (plus mock-transfected COS CM), IL-11 alone, or a combination of IL-11 and IL-3, and the emergence of new blast-cell colonies and their subsequent proliferation in two dishes in each group were recorded daily; the results are presented in Fig 2. Eleven, 12, and 13 blast-cell colonies were identified that later revealed GEMM lineages in cultures containing IL-3 plus mock CM, IL-11, and the combination of IL-3 and IL-11, respectively. The doubling time of individual blast-cell colonies based on the most linear portion of the respective curves was estimated to be 14.0 ± 3.5, 15.2 ± 4.2, and 15.6 ± 3.5 hr in cultures supported by IL-3 plus mock CM, IL-11 alone, and IL-3 plus IL-11, respectively. The doubling time of blast-cell colonies supported by IL-6 plus IL-3 in a simultaneous experiment was 13.6 ± 2.3 hr (data not shown in Fig. 2). The average number of days required for colonies to reach 100 cells was calculated to be 9.1 ± 1.6 days in cultures supported by IL-3 plus mock CM, 10.6 ± 2.0 days in cultures containing IL-11, and 5.0 ± 0.7 days in cultures containing IL-3 and IL-11. Simultaneous studies with a combination of IL-3 and IL-6 revealed the average day to be 5.5 ± 0.8 days (data not shown in Fig. 2). Because the growth rates were not statistically different in these culture systems, these results indicate that the synergistic effect of IL-11 results from a decrease in the time stem cells spend in the dormant state, an effect very similar to that observed with IL-6 or G-CSF.

Comparison of the Replating Potentials of Blast-Cell Colonies. The proliferative potentials of blast-cell colonies that respond to IL-11 and IL-6 were tested by replating experiments. One million day 4 post-5-FU spleen cells were cultured in the presence of IL-3 alone, a combination of IL-3 and

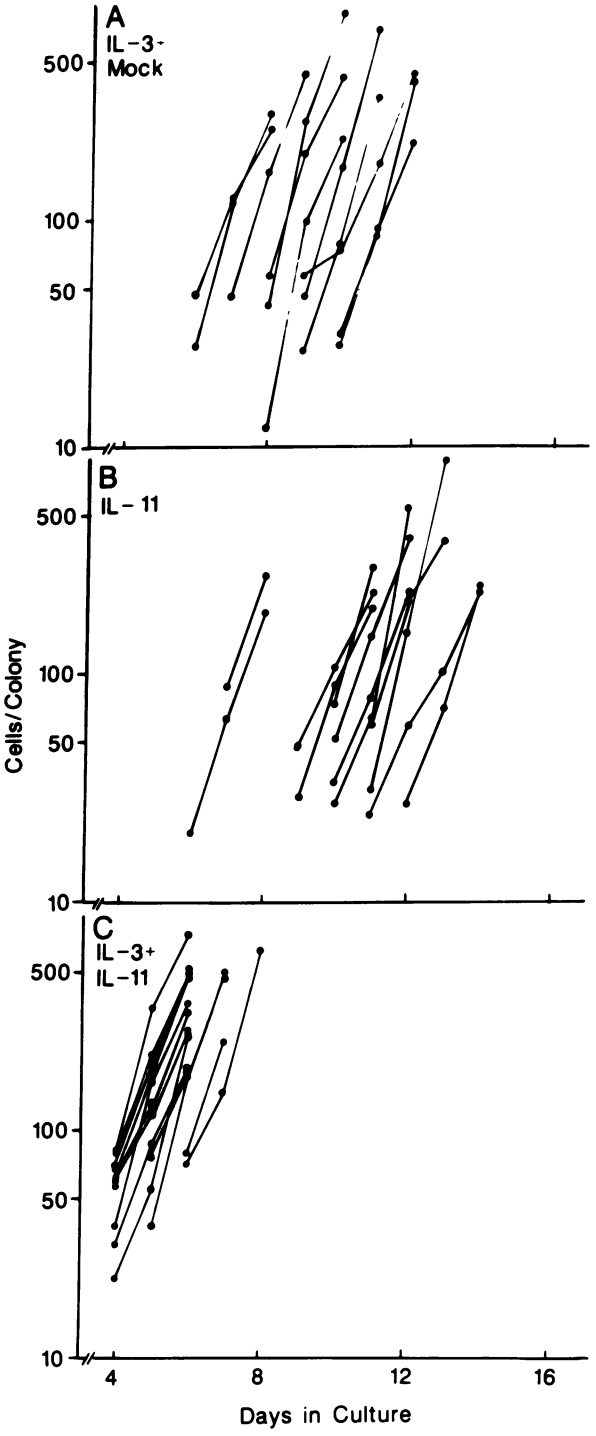

FIG. 2. Graphic presentation of cell-number changes in individual blast-cell colonies that later revealed GEMM expression. Data represent colony formation from two plates each seeded with 1×10^6 spleen cells of 5-FU-treated mice.

IL-6, and a combination of IL-3 and IL-11, and newly appearing small blast-cell colonies consisting of 50–150 cells were picked and replated in secondary culture containing WEHI-3 CM and Ep at 2 units per ml. In cultures containing IL-3 alone, these small blast-cell colonies were identified between day 5 and 10. In cultures containing either IL-11 or IL-6, colony formation was hastened, and therefore blast-cell colonies were identified on day 5 or 6. On day 10 of secondary culture, the number and types of secondary colonies were determined *in situ* by using described criteria (4, 10). The results of replating analyses are summarized in Table 2.

Table 2. Comparison of replating capabilities of multipotential blast-cell colonies supported by IL-3, IL-3 plus IL-11, or IL-3 plus IL-6

Factors in primary culture	Blast-cell colonies, no.	Cells/ blast-cell colonies	Replating efficiency, %	Secondary GEMM colonies/total secondary colonies, %	Secondary GEMM colonies/blast-cell colonies
IL-3	12	76 ± 26	63.1 ± 29.5	13.8 ± 9.9	5.3 ± 4.2
IL-3, IL-11	21	79 ± 29	64.5 ± 29.9	43.7 ± 22.1*	20.1 ± 11.2*
IL-3, IL-6	17	78 ± 30	51.7 ± 22.3	41.4 ± 35.9*	11.9 ± 9.1*

Day 4 post-5-FU spleen cells (1×10^6) were cultured in the presence of IL-3, IL-3 plus IL-11, or IL-3 plus IL-6. From day 5 to day 10 of culture, small blast-cell colonies each containing 50–150 cells were picked from two dishes and transferred to secondary cultures supported by WEHI-3 CM and Ep. Only multipotential blast-cell colonies that exhibited GEMM expression in secondary cultures are tabulated.
*Results are different from IL-3 control at $P < 0.01$ by Student's t test.

Significant variations in the secondary replating efficiencies were seen among individual blast-cell colonies as has been reported (12). There were, however, no significant differences in the replating efficiencies of blast-cell colonies grown in the three different primary culture conditions. Similar to previous observations (12), the percentages of secondary GEMM colonies in secondary colonies and the incidences of secondary GEMM colonies per blast-cell colonies were significantly higher from the primary blast-cell colonies identified in cultures containing IL-3 plus IL-11 or IL-3 plus IL-6 than those seen in cultures containing IL-3 alone. There were no significant differences in these parameters between cultures containing IL-11 plus IL-3 and cultures containing IL-6 plus IL-3 by Student's t test. These results indicated that the synergistic activities of IL-11 and IL-6 are similar and that the increases in the incidences of secondary GEMM colonies may be from shortening of the G_0 period of stem cells during blast-cell colony formation (12).

Replating Studies of Pooled Blast Cells. We have previously shown that IL-6 supports GM colony formation from normal marrow cells (13). In Table 1, we reported that IL-11 also supports GM colony formation. To compare the direct effects of IL-11 and IL-6 on GM colony formation, we used target cells obtained by pooling blast cells from early stages of cultures supported by IL-3. Pooled blast cells are devoid of stromal cells and express very high replating efficiencies. In this study, blast-cell colonies containing 50–150 cells identified in cultures containing IL-3 were picked and pooled and were replated in secondary cultures containing IL-11, IL-6, or IL-3 in the presence of Ep at 2 units per ml. As summarized in Table 3, 80 blast cells yielded 15, 31, and 53 colonies in the presence of IL-11, IL-6, or IL-3, respectively. These data indicate that at least 70% of the blast cells are hemopoietic progenitors. Although the combination of IL-3 and Ep supported formation of a variety of single lineage and multilineage colonies, IL-11 and Ep supported the formation of only macrophage colonies. The combination of IL-6 and Ep supported formation of a similar number of pure macrophage colonies but also neutrophil/macrophage colonies. The macrophage colonies supported by IL-11 were smaller than the macrophage colonies supported by IL-6. These results indicated that IL-11 and IL-6 interact with overlapping, but different, progenitor subsets and that IL-11 preferentially supports the macrophage progenitor population.

Table 3. Cytological analysis of types of secondary colonies derived from pooled blast cells

Lineages expressed in secondary colonies						Secondary colonies, no.		
n	m	Mast	e	E	M	IL-11, Ep	IL-6, Ep	IL-3, Ep
	+					15	15	7
		+				0	0	1
+	+					0	15	18
	+			+		0	1	0
		+	+			0	0	1
+	+	+				0	0	1
+	+			+		0	0	1
+		+	+			0	0	1
+		+		+		0	0	1
+	+	+	+			0	0	10
+	+	+		+		0	0	3
+	+	+			+	0	0	1
+	+			+	+	0	0	1
+	+	+	+	+		0	0	1
+	+	+		+	+	0	0	3
+	+	+	+	+	+	0	0	3
			Total			15	31	53

The colonies in each group were derived from a total of 80 blast cells plated in four dishes. Spleen cells from 5-FU-treated mice were cultured in the presence of IL-3 at 100 units per ml. On day 8 of culture, blast-cell colonies consisting of 50–150 cells (mean 98) were harvested with an Eppendorf pipet and pooled. After washing twice, 20 cells were plated per dish containing IL-11, IL-6 at 50 ng/ml, or IL-3 at 100 units per ml in the presence of urinary Ep at 2 units/ml. Mock CM did not support colony formation. n, neutrophil; m, macrophage; Mast, mast cell; e, eosinophil; E, erythrocyte; M, megakaryocyte colonies.

Effects of Neutralizing Anti-IL-6 Antibody on the Synergistic Effects of IL-11. The difference in the direct colony-supporting ability between IL-11 and IL-6 presented in Table 3 could have resulted from the crude nature of the COS cell CM. Because the synergistic effects of IL-11 on IL-3-dependent proliferation from dormant progenitors are very similar to those of IL-6, there was a remote possibility that transfection with the IL-11 cDNA induced expression of the COS cell IL-6 gene. To exclude this possibility, we used neutralizing anti-IL-6 antibody, which is known to inhibit COS-derived IL-6 in studies of the synergistic effects of IL-11 and IL-6. The results are presented in Table 4. In the presence of IL-6 or IL-11 and in the absence of antibodies,

Table 4. Effects of anti-IL-6 antibody on colony formation by spleen cells of 5-FU-treated mice

Factors	Anti-IL-6 antibody	Colonies, no. Day 8	Colonies, no. Day 16
Ep, IL-3, mock CM	+	14 ± 3	42 ± 6
	−	14 ± 2	39 ± 7
Ep, IL-3, IL-6	+	10 ± 2**	32 ± 4*
	−	51 ± 5	46 ± 8
Ep, IL-3, IL-11	+	54 ± 4	47 ± 3
	−	51 ± 8	45 ± 2

Concentration of factors was as follows: recombinant human Ep, 2 units/ml; IL-3, 100 units/ml; IL-6, 50 ng/ml; and IL-11, 1:100. Data represent mean ± SD of colonies in quadruplicate cultures. Anti-human IL-6 was added to the cultures at a final dilution of 1:200. Rabbit IgG (final dilution, 1:200) was added to the culture as a control. Anti-IL-6 caused significant suppression (*$P < 0.05$, **$P < 0.01$ by Student's t test) of colony formation only in cultures supported by Ep, IL-3, and IL-6.

colony development from day 4 post-5-FU spleen cells was significantly hastened, as indicated by the number of colonies on day 8. When anti-IL-6 antibody was present, the synergistic effects of IL-6 were completely abrogated, whereas the effects of IL-11 were not. The effects of the antibody persisted until day 16. These results excluded the possibility that the apparent synergistic effects in COS cell CM were mediated by IL-6.

DISCUSSION

IL-11 is a cytokine derived from primate bone marrow stromal cells. It was identified by its ability to support proliferation of IL-6-dependent plasmacytoma cell line T1165. Because IL-6 interacts with a variety of target cells within the lymphoid and myeloid lineages, we tested IL-11 for effectiveness in various IL-6-dependent processes. Here we report that the CM of COS cells transfected with IL-11 cDNA augments IL-3-dependent proliferation of multipotential progenitors in culture, an activity originally associated with IL-6. As for IL-6, the mechanism of augmentation appears to be shortening of the G_0 period of dormant stem cells. G-CSF has also been proven effective in augmentation of IL-3-dependent proliferation of early hemopoietic progenitors through shortening of stem cell G_0 period. IL-11 is, therefore, the third factor with the ability to shorten the G_0 period of stem cells. We recently reported that leukemia-inhibitory factor/differentiation-inhibiting activity augments human blast-cell colony formation supported by IL-3 (14). However, studies in our laboratory suggested that leukemia-inhibitory factor/differentiation-inhibiting activity may not shorten the G_0 period (14) and that leukemia-inhibitory factor/differentiation-inhibiting activity did not augment murine IL-3-dependent blast-cell colony formation (data not shown). Because of its effect on stem cells, IL-6 has been shown to increase the numbers of CFU-GEMM (12) and spleen colony-forming cells (15) in suspension cultures containing IL-3 and to facilitate retrovirus-mediated gene transfer to hemopoietic stem cells (15). Because the effects of IL-11 and IL-6 are so similar, we expect that IL-11, as well, may prove useful in manipulation of stem cells for both experimental and clinical purposes.

In addition to the effects on dormant stem cells, all of the synergistic factors appear to control later stages of hemopoietic development. For example, G-CSF is known to be an important growth factor for neutrophil proliferation and maturation. IL-6 stimulates the production of neutrophil/macrophage colony formation in culture (12) and appears important in late megakaryopoiesis and thrombopoiesis (16, 17). In addition, IL-6 is known to stimulate antibody production and synthesis of acute-phase reactants by hepatocytes (18). In contrast to IL-6, IL-11 alone appears relatively specific in supporting macrophage proliferation. However, at least in one culture system, IL-11, like IL-6, acts synergistically with IL-3 in support of megakaryocyte colony formation (8). It appears that G-CSF, IL-6, and IL-11 interact with early and late hemopoietic lineages and that IL-11 and IL-6 may also act within the lymphoid lineages, resulting in stimulation of multiple arms of the host defense system.

The authors thank Mrs. Geneva Winkler for her excellent technical assistance and Dr. Pamela N. Pharr, Mrs. Anne G. Leary, and Mrs. Linda S. Vann for their assistance in the preparation of this manuscript. This work was supported by National Institutes of Health Grant DK32294, the Department of Veterans Affairs, and the Kirin Brewery (Japan). M.O. is a Department of Veterans Affairs Medical Investigator.

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