A RECOMBINANT MURINE GRANULOCYTE/MACROPHAGE (GM) COLONY-STIMULATING FACTOR DERIVED FROM AN INDUCER T CELL LINE (IH5.5)

Functional Restriction to GM Progenitor Cells

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Colony-stimulating factors (CSFs) are glycoproteins that regulate the hematopoietic progenitor cells to proliferate and differentiate. Murine CSFs were well characterized, and four of them, multi-, granulocyte/macrophage (GM)-, granulocyte (G)-, and macrophage (M)-CSF, were purified to homogeneity (1). Multi-CSF acts on various committed hemopoietic progenitor cells as well as pluripotent stem cells, while G-CSF and M-CSF preferentially stimulate the formation of granulocytes and macrophages, respectively. GM-CSF supports the proliferation of granulocyte/macrophage populations and, at very high concentrations, it was also reported (2) to stimulate megakaryocyte (MK), eosinophil (Eo), and erythroid colony formation as well. Recent analyses of complementary DNA (cDNA) encoding murine multi-(3, 4) and GM-CSF (5-7), revealed that these factors belong to distinct molecular species and bear no structural similarity.

We previously reported that when a murine T cell line, 2E10.4.13, was stimulated with PMA, the cells produced at least five hemopoietic activities, including erythroid burst-promoting activity (BPA), erythropoietin (Ep), GM-colony stimulating activity (CSA), MK-CSA, Eo-CSA, and IL-3, in addition to the lymphocyte growth factor, IL-2, even in serum-free medium (8). In ion-exchange HPLC, although all of the hemopoietic activities were more or less associated with IL-3 activity as assayed using an IL-3-dependent cell line, the distinct peak of each activity existed also in the region where no IL-3 activity was detected. Therefore, we concluded that CSF activities mediated by the distinct molecules from IL-3 were produced by the T cell line, which prompted us to study further the hemopoietic factors generated from this cell line by using a molecular cloning technique.

We here report the functional characterization of hemopoiesis of recombinant...
GM-CSF (rGM-CSF) derived from IH5.5 cells, which were cloned from the same parental cell line as 2E10.4.13.

Materials and Methods

Mice. Inbred female ICR, BALB/c, and BDF; mice, aged 10–15 wk, and pregnant ICR mice were purchased from the Shizuoka Experimental Animal Center (Shizuoka, Japan).

Inducer T Cell Line, IH5.5. A continuous cell line, IH5.5, was obtained in the process of cloning the IL-2-dependent large granular lymphocyte (LGL) line, PEC-1, from BALB/c mouse peritoneal exudate cells injected with syngeneic RL21 leukemia cells (9). The majority of PEC-1 line cells showed LGL morphology, and the cloning by limiting dilution resulted mostly in typical LGL clones with NK activity. However, a small number of colonies with distinct features were also obtained from LGL colonies, clone IH5.5 being one of them. Clone IH5.5 lacked cytoplasmic granules and serological analysis indicated the phenotype of Thy-1+, Lyt-1+, Lyt-2-, T200+. They were also distinct from RL51 cells used for the in vivo stimulation, in that RL51 cells were Thy-1+, Lyt-1+, Lyt-2- and did not produce lymphokines. The β gene of the T cell receptor was productively rearranged in the IH5.5 clone. The rearrangement pattern, however, was distinct from parental PEC-1, as well as the LGL clones derived thereof, making it very unlikely that they were directly derived from LGL. Such T cell lines were obtained only in the process of cloning, and their growth was independent of IL-2. A minor fraction of inducer type T cells seems to have coexisted in an original PEC-1 line and their outgrowth in mass cultures seems to have been inhibited by the majority of LGL in the continuous line.

Preparation of Crude Conditioned Medium. IH5.5 cells were maintained in RPMI 1640 tissue culture medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% FCS (Flow Laboratories, Inc., McLean, VA), as described previously (9). Conditioned medium was prepared as follows: The cells were incubated with 50 ng/ml of PMA (Sigma Chemical Co., St. Louis, MO) and 1% PHA M (Difco Laboratories, Inc., Detroit, MI) in the absence of FCS. After 48 h of incubation, the supernatant was harvested, filter-sterilized, and used as the crude conditioned medium, designated crude-CM.

IL-2 and IL-3 Assays. IL-2-dependent (CTLL-2) and IL-3-dependent cell lines (FDC-P1) were used for the assays of IL-2 and IL-3 activities. In brief, 10⁴ cells were suspended in 0.1 ml RPMI 1640 medium supplemented with 10% FCS then plated in a microtiter well to which we added 0.1 ml of the sample to be tested. After incubation for 18 h, 0.5 μCi of [³H]thymidine (sp act, 15 Ci/mmol) in 20 ul RPMI 1640 medium was added to each well, then incubation was continued for a further 8 h. The radioactivity incorporated into the cells was counted with a scintillation counter (Aloka Co., Ltd., Tokyo, Japan).

Chromatography. A 40-fold concentrate of crude-CM was applied to a 2 x 60 cm column of Sephadex G-100 (Pharmacia Fine Chemicals, Uppsala, Sweden) that had been equilibrated with 0.05 M Tris-HCl buffer, pH 7.4, containing 150 mM NaCl, 10 mM CaCl₂, 2H₂O, and 0.05% PEG. The column was flushed with the same buffer at 4°C, and 2-ml fractions were collected and assayed. In this column, BPA, GM-CSA, MK-CSA, Eo-CSA, IL-2, and IL-3 were eluted at almost the same region immediately after the major protein peak. These active fractions were pooled, concentrated 15-fold, and subjected to HPLC. Ion-exchange HPLC was carried out as follows: The concentrate was dialyzed against 0.02 M Tris-HCl, pH 7.4, containing 0.02% Tween 20 (Wako Pure Chemical Industries, Tokyo, Japan) and was applied to a TSK-Gel IEX-500 QAE-2SW (5 μm, 4.6 x 250 mm; Toyo Soda, Tokyo, Japan) that had been equilibrated with the same buffer. Elution was first performed at room temperature for 10 min with the starting buffer, then with the starting buffer and a linear gradient of 0 M–0.5 M NaCl for 75 min, and finally with the starting buffer containing 0.5 M NaCl for 10 min. The flow rate was 0.5 ml/min throughout these procedures. The effluent was collected as 0.3-ml fractions per tube and was used for the assays after a threefold dilution with DME.

Cell Preparations. Bone marrow cells were prepared from femurs of ICR and BALB/c mice, and phagocytic cells were eliminated by treatment with carbonyl iron (GAF Corp.,
New York) as previously described (10). The nonphagocytic cells were used for granulocyte/macrophage, megakaryocyte, eosinophil and erythroid colony assays. Fetal liver cells were collected from fetuses of ICR mice at 12 days of gestation and were dissociated mechanically in DME (Gibco Laboratories) by successive passages through 22- and 27-gauge needles. The nonadherent, nonphagocytic cells were used for the erythroid colony assays.

10- to 15-wk-old female BDF I mice were administered 5-fluorouracil (5-FU) (Adria Laboratories, Inc., Columbus, Ohio) through the tail vein at a dosage of 150 mg/kg body weight. Spleen cells were harvested 4 d later, and we prepared single-cell suspensions from three to five mice.

Construction of Plasmid and Preparation of Recombinant CSF. The mouse T cell line IH5.5 (clone Al) was stimulated with 1% PHA and 50 ng/ml of PMA. The cells were harvested 6 h and 9 h after stimulation. Total cellular RNA was extracted from the cells by using the guanidinium thiocyanate method (11), and poly(A)$^+$ RNA was selected by oligo(dT) cellulose column chromatography. Aliquots of the two induced mRNA samples were then pooled for the construction of a cDNA library. The cDNA library was constructed by using the pcDV1 vector-primer and pL1 linker fragment according to the procedures of Okayama and Berg (12). About 6 µg of poly(A)$^+$ RNA yielded $\sim 6 \times 10^5$ independent transformants.

Two synthetic oligodeoxynucleotides, 5'-TGG AAG CAT GTAGAG GCC-3' and 5'-CTG GCC TGGGCT TCC TCA-3', were synthesized according to the available sequence of GM-CSF from murine lung (5). Two probes were used to screen $\sim 3 \times 10^9$ colonies of the cDNA library transferred onto 541 filters from Whatman Inc. (Clifton, NJ). Hybridization conditions used overnight at 50°C were: 6X SSC (1X SSC = 0.15 M NaCl and 0.015 M sodium citrate, pH 7.2), 1X Denhardt's (0.02% Ficoll, 0.02% polyvinyl-pyrrolidone, and 0.02% BSA), 0.1% SDS, and 50 µg/ml of denatured salmon sperm DNA. The filters were washed at 50°C with two changes of 6X SSC for 30 min. One clone out of 3 $\times 10^4$ clones gave a positive signal with two probes and was designated pHS mGM-CSF. The plasmid DNA pHS mGM-CSF, which promoted the expression of mGM-CSF in mammalian cells, was transfected into COS-1 cells by the DNA-calcium phosphate coprecipitation technique (13). After transfection, the medium was replaced with serum-free DME. After 72 h, we collected the medium and characterized the translation product, termed rGM-CSF, by using various colony assay systems.

Colony Assays. GM and Eo, and MK colonies were assayed by soft-agar culture methods described elsewhere (14), using ICR and BALB/c mice bone marrow cells, respectively. The agar gel was fixed and stained (10). Cell aggregates containing $\geq 50$ naphthol AS-D chloroacetate-/α-naphthyl butyrate esterase-staining positive cells (14), $\geq 4$ acetylcholinesterase-staining positive cells (15), and $\geq 50$ Bieblich scarlet-staining positive cells (16) were scored as the respective GM, MK, and Eo colonies. The erythroid colony assay was carried out by the methylcellulose culture method (8), using both ICR mouse bone marrow (10$^5$ cells/ml) and fetal liver cells (10$^5$ cells/ml). At the end of 72 h of incubation, 1 U/ml of purified human urinary Ep with a specific activity of 81,600 U/mg protein (the kind gift of Dr. M. Ueda, Snow Brand Milk Co. Ltd., Tochigi, Japan) was overlaid on the methylcellulose culture. After 8 d, cell aggregates containing $\geq 50$ erythroid cells, or $\geq 3$ compact subcolonies of erythroid cells, were counted as erythroid bursts. Then, the colonies were picked up from the methylcellulose culture, spun in a Shandon cytocentrifuge, and then the lineage expression of colonies was investigated.

Colony assays using spleen cells from 5-FU-treated mice were carried out as follows: Methylcellulose culture was performed by using a modification of the technique described previously (17). Spleen cells (1.2 $\times 10^6$ cells/ml) from 5-FU-treated mice were incubated in 1 ml of culture medium containing α-medium (Gibco Laboratories), 1.2% methylcellulose (Fisher Scientific Co., Pittsburgh, PA), 50% FCS, 1% deionized BSA (Sigma Chemical Co.), 0.1 mM 2-ME (Sigma Chemical Co.) and samples. On day 12 of incubation, the number of colonies was scored, lifted from the methylcellulose cultures using a 3-µl Eppendorf pipet under microscopic visualization, and then individually suspended in 0.1
TABLE I
Hemopoietic Activities and Lymphokines Detected in the Medium Conditioned by IH5.5 Cells Stimulated with PMA and PHA

| Medium conditioned with | Number of colonies* | Radioactivity incorporated (cpm)$^\dagger$ |
|-------------------------|---------------------|-------------------------------------|
| Cells                   | GM  | Eo  | MK  | E   | IL-2 | IL-3 |
| Cells + PMA-PHA         | 48 ± 5 | 0   | 35 ± 3 | 26 ± 3 | 59,734 | 10,150 |

Crude serum-free medium conditioned by IH5.5 cells stimulated with or without the combination of PMA and PHA.

* GM, Eo, or MK per 5 $\times$ 10$^4$ bone marrow cells in soft agar cultures; E per 10$^5$ bone marrow cells in methylcellulose cultures. Data are shown as the mean ± SD of triplicate cultures.

† Radioactivity incorporated into CTLL-2 or FDC-P2 cells. Normal bone marrow cells were incubated with the samples.

§ On day 7 of soft agar culture, colonies were fixed and stained, then counted. Erythroid colony assay was performed by the methylcellulose culture method, in which purified Ep was added after 72 h and the colonies were scored on day 8 of culture. Data are shown as the mean ± SD of triplicate cultures.

ml of α-medium. The cell suspensions were spun in a cytocentrifuge from Shandon Southern Instruments Inc. (Sewickley, PA) and stained with May-Grunwald-Giemsa.

Spleen cells from 5-FU-treated mice were cultured to obtain the blast cell colonies (17). Briefly, spleen cells were incubated in the methylcellulose culture medium as described above, by the addition of PWM-stimulated spleen cell–conditioned medium (PWM-SCM) (18). On day 7 of incubation, blast cell colonies containing 20–100 cells were lifted from the methylcellulose medium and pooled. The cells were washed twice with α-medium to remove the effect of PWM-SCM and were replated, at a concentration of 300 cells or a single cell per ml, into a 35-mm culture dish containing 1 ml of methylcellulose medium supplemented with each sample. Transfer of a single cell was performed using a micro-manipulator (Narishige Scientific Instrument Laboratory, Tokyo, Japan), as described previously (17). After 7 d of incubation, all cell aggregates were picked up from methylcellulose culture, spun in a cytocentrifuge (Shandon Southern Instruments Inc.), and stained with May-Grunwald-Giemsa. The size of small colonies (<500 cells) was estimated in situ, and that of larger colonies by using a counting chamber.

Results

Production of Multiple Hemopoietic Factors from a Cloned T Cell Line, IH5.5. As shown in Table I, BPA, GM-CSA, and MK-CSA, as well as IL-2 and IL-3 activities, were detected in the crude-CM of IH5.5 stimulated with PMA and PHA, while no Eo-CSA was detected. IH5.5 cells ceased proliferation when we added either PMA or PHA, or significantly increased in cell size almost immediately when both were added. Since IL-3 was shown to have multihemopoietic activity (19), fractionation of each hemopoietic activity in crude-CM was performed with reference to IL-3 activity. When the conditioned medium was fractionated by ion-exchange HPLC with a linear gradient of NaCl, hemopoietic factors were eluted in three distinct regions of 0.02–0.1 M, 0.18–0.25 M, and 0.33–0.38 M NaCl (Fig. 1). IL-3 activity (0.02–0.1 M) was associated with BPA, as well as with a minor peak of GM- and MK-CSA, whereas the major peaks of GM- and MK-CSA were detected in the region that is distinct from IL-3 (0.33–0.38 M). These results indicated that the T cell line produced GM- and MK-CSF distinct from IL-3.
**Selective GM Colony-stimulating Activity of rGM-CSF.** Gene cloning of murine GM-CSF (mGM-CSF) has already been reported (5-7) using lung cells from mouse injected with bacterial endotoxin and two T cell lines, EL-4 stimulated with PMA and LB-3 stimulated with Con A. Using a portion of the sequence of the GM-CSF, a candidate gene, pHS mGM-CSF was cloned from a cDNA library of the T cell clone, IH5.5, as described in Materials and Methods. Serum-free conditioned medium was prepared from COS-1 cells transfected with pHS mGM-CSF and was used to characterize the activity of the translation products, rGM-CSF. Table II shows the results of colony assays using bone marrow cells prepared from normal mice. When the crude-CM of IH5.5 stimulated with PMA and PHA was added to soft-agar or a methylcellulose culture system containing bone marrow cells, GM, MK, and erythroid colonies formed. rGM-CSF, on the other hand, induced only the formation of granulocyte/macrophage colonies. In the case of the methylcellulose culture using fetal liver cells, we saw very few erythroid bursts in addition to nonerythroid colonies in the presence of rGM-CSF and highly purified Ep. By contrast, the crude-CM stimulated the formation of many erythroid bursts, while we saw no erythroid bursts in the presence of purified Ep alone (Table III). To further distinguish the cells in the nonerythroid colonies from bone marrow and fetal liver cells, these colonies were picked up from the methylcellulose medium and stained with May-Grünwald-Giemsa. All of the nonerythroid colonies lifted from the cultures containing rGM-CSF consisted of granulocytes and/or macrophages, and no other cell types were found. On the other hand, when crude-CM was used, nonerythroid colonies contained a variety of hemopoietic cells, including neutrophils, macrophages,
TABLE II

Morphologic Types of Colonies Derived from Normal Bone Marrow Cells

| Stimulus*       | Concentration | Number of colonies† |
|-----------------|---------------|---------------------|
|                 | (%)           | GM      | Eo     | MK      | E       |
| rGM-CSF         | 10            | 23 ± 5  | 0      | 0       | 0       |
|                 | 20            | 46 ± 6  | 0      | 0       | 0       |
| Mock-COS-SUP    | 10            | 0       | 0      | 0       | 0       |
|                 | 20            | 0       | 0      | 0       | 0       |
| Crude-CM        | 10            | 33 ± 4  | 0      | 23 ± 5  | 19 ± 5  |
|                 | 20            | 56 ± 9  | 0      | 61 ± 6  | 68 ± 8  |

Normal bone marrow cells were incubated with rGM-CSF, mock-COS-SUP, or crude-CM. On day 7 of soft agar culture, colonies were fixed and stained, and then scored. Erythroid colony assay was performed by the methylcellulose culture method, in which purified Ep was added after 72 h. On day 8 of culture, colonies were scored, picked up, and then stained with May-Grünwald-Giemsa. Data are shown as the mean ± SD of triplicate cultures.

* rGM-CSF, supernatant of COS-1 cells transfected with plasmid-carrying GM-CSF-cDNA; mock-COS-SUP, supernatant of mock-transfected COS-1 cells; crude-CM; crude conditioned medium of IH5.5 cells.

† GM, Eo, or MK per 5 × 10⁴ bone marrow cells in soft agar cultures; E per 10⁴ bone marrow cells in methylcellulose cultures.

TABLE III

Morphologic Types of Colonies Derived from Normal Fetal Liver Cells

| Stimulus*       | Concentration | Ep‡ | Number of colonies§ |
|-----------------|---------------|-----|---------------------|
|                 | (%)           | Eo  | Nonerythroid | Erythroid |
| rGM-CSF         | 10            | –   | 36 ± 9         | 0         |
|                 | 20            | +   | 41 ± 6         | 2 ± 2     |
| Mock-COS-SUP    | 10            | –   | 63 ± 5         | 0         |
|                 | 20            | +   | 58 ± 10        | 3 ± 1     |
| Crude-CM        | 10            | –   | 0               | 0         |
|                 | 20            | +   | 0               | 0         |
| Ep              | 1 U/ml        | –   | 0               | 0         |
|                 | +             | 0               | 0         |

Normal fetal liver cells were incubated with rGM-CSF, mock-COS-SUP, crude-CM, or purified Ep, with (+) or without (–) the addition of purified human urinary Ep (1 U/ml) as an overlayer after 72 h. On day 8 of culture, colonies were counted, picked up, and then stained with May-Grünwald-Giemsa. Data are shown as the mean ± SD of triplicate cultures.

* See the footnote for Table II.

‡ Ep, 1 U/ml purified human urinary Ep.

§ Nonerythroid or erythroid colonies per 5 × 10⁴ cells in methylcellulose cultures.
Spleen cells of 5-FU-treated mice were cultured with rGM-CSF, mock-COS-SUP, crude-CM, or PWM-SCM. On day 7 of culture, after counting the number of colonies, each colony was picked up and stained with May-Grünwald-Giemsa, and the morphologic type of the cells was determined.

*A See the footnote of Table II. PWM-SCM: pokeweed mitogen-stimulated spleen cell conditioned medium.

† Number of colonies per 1.2 × 10⁶ spleen cells derived from 5-FU-treated mice. GMM, granulocyte/macrophage-megakaryocyte colonies. Data are shown as the mean ± SD of triplicate cultures. 4N, neutrophils.

megakaryocytes, and erythroblasts. In neither assay described above could the formation of colonies be observed by the addition of mock-COS-SUP. These results showed that rGM-CSF was functionally distinct from the IL-3. Indeed, rGM-CSF did not support the growth of the IL-3-dependent cell line, FDC-P2.

rGM-CSF Does Not Act on Pluripotent Stem Cells. To determine the target of rGM-CSF, a more critical colony assay was performed in which 5-FU-treated mouse spleen cells were used as target cells. On day 12 of methylcellulose culture, the colonies were picked up and stained with May–Grünwald–Giemsa (Table IV). When PWM-SCM that also contained IL-3 was added to the culture, we found a variety of hemopoietic cells, including neutrophils, macrophages, megakaryocytes, erythroblasts, and mast cells, in the developed colonies. The crude-CM of stimulated 1H5.5 cells showed similar biological activities. In contrast, rGM-CSF exhibited no stimulatory activities for the formation of colonies from 5-FU-treated mouse spleen cells, indicating that rGM-CSF could not act on 5-FU-resistant pluripotent stem cells.

Development of GM-Colonies from Blast Cells by rGM-CSF. Further investigation was carried out by blast colony assay techniques. Spleen cells from 5-FU-treated mice were incubated in methylcellulose culture medium supplemented with PWM-SCM. After 7 d, blast colonies were picked up and pooled. The pooled cells were replated at a concentration of 300 cells/ml into a new methylcellulose culture medium containing either PWM-SCM, rGM-CSF, or mock-COS-SUP, followed by the incubation for 7 d (Table V). May–Grünwald–Giemsa staining revealed that the colonies formed by rGM-CSF were composed preferentially of neutrophils and/or macrophages, whereas various types of cells other than neutrophils and macrophages were also seen in PWM-SCM–stimulated colonies. Mock-COS-SUP showed no stimulatory activities. Single cells in blast colonies were transferred to the dishes with rGM-CSF by using a micromanipulator (Table VI). On day 7 of culture, all the cell aggregates formed were lifted from the methylcellulose medium, centrifuged, and stained (Fig. 2). From 103 cultures of single cells, 67 proliferating clones developed. Among them, 43 colonies containing 7–8,500 cells were composed exclusively of macrophages, 10 colonies containing 500–8,500 cells consisted of neutrophils, and there were 14 colonies

| Stimulus* | Number of colonies† | Lineages§ |
|-----------|---------------------|-----------|
|           | GM | MK | GMM | N | M | Eo | MK | E | Mast |
| rGM-CSF   | 1 ± 1 | 0 | 0 | + | + | - | - | - | - |
| Mock-COS-SUP | 0 | 0 | 0 | - | - | - | - | - | - |
| Crude-CM  | 10 ± 2 | 2 ± 2 | 2 ± 1 | + | + | - | + | - | + |
| PWM-SCM   | 18 ± 2 | 6 ± 1 | 6 ± 2 | + | + | + | - | - | + |
TABLE V
Morphologic Types of Colonies Formed by Replating Blast Cells Derived from 5-FU-treated Mouse Spleen Cells

| Stimulus     | Number of colonies | Lineages |         |         |         |         |         |
|--------------|--------------------|----------|---------|---------|---------|---------|---------|
|              | GM     | MK     | GMM    | Mast   | N      | M      | E      |
| rGM-CSF      | 146 ± 16 | 0      | 0      | 0      | +      | +      | -      |
| Mock-COS-SUP | 0      | 0      | 0      | 0      | -      | -      | -      |
| PWM-SCM      | 37 ± 7  | 3 ± 1  | 4 ± 1  | 1 ± 1  | +      | +      | +      | -      | -      |

See footnotes for Tables I, II, and IV. Spleen cells (1.2 × 10⁶ cells/ml) from 5-FU-treated mice were cultured with PWM-SCM. On day 7 of incubation, blast cell colonies were pooled and replated at a rate of 300 cells/ml into the methylcellulose medium containing rGM-CSF, mock-COS-SUP, or PWM-SCM. After 7 d of culture, colonies were counted and stained with May-Grünwald-Giemsa. Data are shown as the mean ± SD of triplicate cultures.

TABLE VI
Morphologic Types of Colonies Formed by Replating Single Blast Cells Derived from 5-FU-treated Mouse Spleen Cells

| Number of cells | Number of colonies | Neutrophils in each NM colony* |
|-----------------|--------------------|--------------------------------|
|                 | N     | M     | NM    | (%)                           |
| 7-100           | 0     | 9     | 1     | 4.8                           |
| 100-999         | 3     | 19    | 0     |                               |
| 1,000-4,999     | 5     | 12    | 6     | 16.0, 32.8, 33.6, 59.2, 64.8, 72.0 |
| 5,000-9,999     | 3     | 2     | 3     | 4.0, 6.4, 21.6               |
| 10,000-15,000   | 0     | 0     | 4     | 5.6, 13.6, 16.0, 21.2         |
| Total           | 10    | 43    | 14    |

See footnote to Table IV. Spleen cells (1.2 × 10⁶ cells/ml) from 5-FU-treated mice were cultured with PWM-SCM. On day 8 of incubation, blast cell colonies were pooled and single blast cells were replated into the methylcellulose medium containing rGM-CSF. After 7 d of culture, colonies were scored and picked up. Then the cells in each colony were counted and stained with May-Grünwald-Giemsa for differential counting.

* Range of the number of cells contained in a single colony. See footnote to Table IV.

Table VI contains 33-15,000 cells of neutrophils and macrophages together (Table VI). In this culture, we saw no other hemopoietic lineage cells. The percentage of neutrophils in the neutrophil/macrophage mixed colonies varied considerably from colony to colony, ranging from 4.8 to 72.0%. The numbers of cells contained in the colonies were also distributed in a wide range. No relation was found between the size of colony and the percentage of neutrophils in individual colonies.

Discussion

We performed the present study to define the in vitro hemopoietic activity of GM-CSF produced by an inducer T cell line. A gene for GM-CSF was cloned from a cDNA library of a T cell clone, IH5.5, using a part of the DNA sequence of a GM-CSF gene obtained from murine lung cells (5), and rGM-CSF was obtained by the expression of COS-1 cells.
rGM-CSF was first tested in standard colony assay systems of soft agar and methylcellulose by using normal bone marrow cells. The results indicated that only neutrophil/macrophage colonies were formed by rGM-CSF. In a subsequent experiment, fetal liver cells were used as target cells. The rGM-CSF alone failed to stimulate the formation of erythroid bursts. Combined stimulation with rGM-CSF and purified Ep led to the formation of only a few erythroid bursts, compared with the combination of crude-CM and Ep. It thus seems that rGM-CSF does not have significant BPA activity. Preliminary experiments using a larger amount of purified rGM-CSF derived from Escherichia coli revealed the same results mentioned above (data not shown).
Treatment of mice with a high dose of 5-FU has been reported (20, 21) to result in a significant enrichment of the more primitive hematopoietic stem cells. We used this system to study the action of rGM-CSF on pluripotent hematopoietic progenitors. When spleen cells derived from 5-FU-treated mice were incubated with crude-CM or PWM-SCM, GM and MK, as well as mixed colonies, were formed. In contrast, rGM-CSF was completely inactive on such spleen cells. We have already reported (22, 23) that IL-3 supported the colony growth from 5-FU-treated mouse spleen cells. The rGM-CSF, however, can stimulate colony formation by blast cells that had been grown in the presence of PWM-SCM from 5-FU-treated mouse spleen cells. These colonies consisted solely of neutrophils and/or macrophages, whereas control cultures supplemented with PWM-SCM induced various types of colonies (neutrophil, macrophage, megakaryocyte, eosinophil, and mast cell). We did not see eosinophils in either assay. We checked the dose-response curve for the colony formation stimulated by rGM-CSF ranging from 2.5 to 10%. The colony size increased with increase in the concentration of rGM-CSF, although the proportion of neutrophil colonies to macrophage colonies was constant. It could either be due to selective GM-CSF receptor distribution or to intrinsic programming for differentiation in the target cells. A binding assay of radiolabeled rGM-CSF is in progress. rGM-CSF supported the growth of GM colonies from single cells isolated from blast cell colonies by micromanipulation. It thus indicated that rGM-CSF acted directly on committed precursors without the accessory cells and supported the differentiation into mature neutrophils and macrophages. The basis for the lineage restriction of rGM-CSF is not known yet.

The single lineage colonies were not always smaller than bilineage colonies. For example, one of the neutrophil/macrophage colonies contained only 33 cells, and a pure neutrophil colony contained 8,500 cells. This shows that the number of cell divisions occurring after commitment is quite variable. This concept is not in agreement with the model that exhibits a relatively constant number of cell doubling during the maturation process. Lineage restriction and loss of proliferative capacity of individual progenitors may be depicted as separate stochastic processes. We also showed that the ratios of neutrophils and macrophages in the mixed colonies derived from single cells in the presence of rGM-CSF varied greatly, ranging from 4.8 to 72.0%. This means that GM-CSF is permissive for the proliferation of neutrophils and macrophages, but it does not determine the differentiation pathway. How the differentiation programs progress in the presence of hematopoietic growth factors remains to be discovered.

In conclusion, rGM-CSF obtained from a T cell line was confirmed to preferentially and directly act on committed GM progenitor cells without effecting either pluripotent stem cells or progenitors of hematopoietic cells other than neutrophils and macrophages.

Summary

The cDNA for the murine granulocyte/macrophage colony-stimulating factor (GM-CSF) was cloned from a cDNA library obtained from a murine T cell line, IH5.5, by using two synthetic probes that encoded two parts of the GM-CSF from murine lung. The cDNA inserted into the plasmid vector pcDV1 was transfected into monkey COS-1 cells and the conditioned medium was used to
investigate the hemopoietic activities of the resultant product, recombinant GM-CSF (rGM-CSF), by means of various colony assays. rGM-CSF stimulated only neutrophil/macrophage colonies in the cultures of murine normal bone marrow and fetal liver cells. No other colony stimulating activities (CSA) were seen in the preparation including burst-promoting activity, eosinophil-CSA, megakaryocyte-CSA and mast cell-CSA. rGM-CSF could not support colony formation of 5-fluorouracil-treated mouse spleen cells, in which only the primitive population of stem cells survived. However, after culture of these cells with PWM-spleen cell-conditioned medium (PWM-SCM), the colonies consisting of blast cells were formed. These blast cells could now be induced to form neutrophil/macrophage colonies in the presence of rGM-CSF. Pure neutrophil colonies, pure macrophage colonies, as well as mixed neutrophil/macrophage colonies, were formed from these single blast cells in the presence of rGM-CSF by micromanipulation. rGM-CSF did not act on pluripotent hemopoietic stem cells, but did act directly and selectively on neutrophil/macrophage progenitors. Moreover, striking heterogeneities were noted in the size of the colonies and the proportion of components. GM-CSF is, therefore, considered to play a noninstructive role in the differentiation of the GM pathway.

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