Production of Granulocyte-Macrophage Colony-Stimulating Factors (GM-CSF) by Various Mammalian Cell Lines Cultured in a Protein-Free Synthetic Medium

Kazuko Tsuneoka and Mikio Shikita

National Institute of Radiological Sciences, Anagawa 4-chome, Chiba-shi 260, Japan

ABSTRACT. Thirteen cell lines, all of which had been adapted to grow in a chemically defined medium, produced colony-stimulating factor (CSF), but the production rates of the factor varied considerably among the cell lines. Rat liver parenchymal, monkey kidney, and rat spleen plasma cell lines were comparable to L-P3 cells in their CSF-producing activity. Isoelectrofocusing and gel-filtration chromatography in the presence of 6 M guanidine HCl showed that the CSFs of the liver and kidney cell lines resembled L-P3 CSF, whereas the CSF of spleen cells differed from the other CSFs in its molecular properties. In addition, the CSF of the spleen cell line predominantly stimulated the formation of granulocytic colonies, but the L-P3 CSF and other CSFs mainly enhanced monocytic colony formation.

Myeloid stem cells themselves do not proliferate spontaneously, when they are isolated from hematopoietic tissues and dispersed in a medium supplemented with essential nutrients and serum. A specific glycoprotein, called colony-stimulating factor (CSF), is required for them to proliferate (11). In the absence of this factor, the stem cells cannot even survive under the culture conditions (10). The activity of CSF has been detected in various tissue extracts (17) as well as in media conditioned against cells of various organs (13) or against various cultured cell lines (3, 14, 16, 18). Because it exists in a number of sources, the specificity of its action as a regulator of the proliferation of myeloid stem cells has been doubted (8, 15). Recent reports on its inefficacy in stimulating granulopoiesis in long-term cultures of bone marrow cells have raised further arguments about its role as a true granulopoietin (2, 24). This led us to examine the generality of this factor as a product of various mammalian cell lines which had been adapted to grow in a chemically defined medium. It has been speculated that a principle produced by these cells eventually stimulates the proliferation of myeloid stem cells as well as these cells themselves. However, the rate of production of CSF varies considerably from one cell line to another, and is seemingly unrelated to the growth rate of these cells in culture. We also report that there are two distinct types of CSF, one that predominantly produces granulocytes and another that predominantly produces monocytes.

Abbreviation: CSF, colony-stimulating factor; PEG, polyethylene glycol 4000

MATERIALS AND METHODS

Cells. The cells have been maintained in a synthetic medium DM-160 (Kyokuto Pharmaceutical Industry, Tokyo) (5, 6, 7, 20) in the laboratory of Drs. Katsuta and Takaoka, Dokkyo University, School of Medicine, Tochigi. Routinely, the cells have been grown in a stationary culture at 37°C on the surface of slanted test tubes containing 1.5 ml of the nutrient solution. The medium was conditioned against grown cells (approximately 10^6 cells/tube) for 3 days then frozen and stored. In the preparative experiment, cells were grown in several tubes as above for 3 or 4 days. The conditioned medium was harvested repeatedly from the same cultures and pooled in the amounts of 120 ml (L·P3), 30 ml (JTC-25·P3), 33 ml (RSP-2·P3) and 65 ml (JTC-12·PC). Protein concentration of the cell-conditioned medium was determined by the method of Bradford (1) with Coomassie brilliant blue G 250 (Bio-rad, Richmond, CA) after dialysis of the medium against 0.15 M NaCl containing 5 mM Na phosphate (pH 7.4) and 0.05% polyethylene glycol (PEG).

Chromatography and isoelectrofocusing. In the preparative experiment, the cell-conditioned medium was concentrated and dialyzed against 0.05% PEG, then centrifuged at 3,000 rpm for 15 min to remove precipitates, after which it was isoelectrofocussed in a 20-ml glass tube as reported previously (22). A sample of the focused CSF was treated with neuraminidase (2 μ g/ml) for 3 h at 37°C in 0.1 M acetate buffer at pH 5.5. The CSF solutions, before and after the enzyme treatment, were mixed with appropriate amounts of solid guanidine HCl and then dialyzed against a large volume of 6 M guanidine HCl containing 0.15 M NaCl and 5 mM Na phosphate (pH 7.4). A portion (0.5 ml) of each CSF solution was applied to a Toyo-Pearl HW 55F column (0.9×60 cm), which had been equilibrated with the guanidine solution. Twelve-drop fractions (about 0.38 ml) were collected at a rate of 2 ml/h. Guanidine was removed from the eluate by dialysis.

The gel-filtration column was calibrated with standard proteins, which were run separately as above. Blue dextran and cytochrome *c* were added to each run of the CSF solution as internal standards.

Assay of CSF activity. The CSF assay was the same as reported previously (21). Briefly, femoral marrow cells of male C3H mice (aged about 3 months) were cultured for a week in McCoy's 5A medium containing 0.32% agar, 20% horse serum, and various amounts of CSF. The initial cell density was 1×10^5 /dish. One unit of CSF represents one colony in the culture. The colonies produced were counted under a magnification of 40. Some cultures were treated with 30% acetic acid in ethanol and washed 3 times with decreasing concentrations of aqueous ethanol according to the method described by Wu and Yunis (25). The contracted agar layers were removed intact from the petri dishes and transferred to glass plates, and dried thoroughly at 37°C. Cells embedded in the thin film of dried agar were then stained with hematoxylin and examined microscopically at 200x magnification. Granulocyte colonies were distinguished easily from monocyte colonies by the size of the cells and by the characteristic doughnut- or horseshoe-like shape of the cell nucleus. Colonies containing both granulocytes and monocytes (mixed type colonies) were less than 20% of the total number and were included in the count of granulocytic colonies.

RESULTS AND DISCUSSION

Production of CSF by various mammalian cell lines growing in a protein-free synthetic medium. The results of the present study show that all the cell lines adapted to grow in a protein-free medium produced CSF(s), although the rate of production of the factor(s) varied markedly among the lines (Table 1). Unlike the macrophage growth

TABLE 1. PRODUCTION OF CSF BY MAMMALIAN CELL LINES
GROWING IN A PROTEIN-FREE MEDIUM

Cell line	Dialyzed cell-conditioned medium	
	Protein ($\mu\text{g/ml}$)	CSF (units/ml)
JTC-21-P3 (rat liver parenchymal cells)	100	330 ^a
JTC-23-P3 (//)	3	760
JTC-25-P3 (//)	1	550(3000) ^b
JTC-16-P3 (rat ascites hepatoma cells)	6	30
JTC-12-P3 (monkey kidney cells)	24	615(1140)
MDCK-P3 (dog kidney cells)	47	45
MDBK-P3 (bovine kidney cells)	5	15
L-P3 (mouse fibroblasts)	4	1100(3625)
L-P3 Co-3 (⁶⁰ Co resistant substrain of L-P3)	24	1200
RLC-10-P3 (normal rat liver cells)	16	80
M-P3 (rat liver cells)	9	70
RSP-2-P3 (rat spleen plasma cells)	58	500(1100)
HeLa-P3 (human uterus carcinoma cells)	3	20

^a Average of three assays. There was no spontaneous colony formation under the assay conditions used.

^b Figures in parentheses represent the CSF activity obtained in the preparative experiment.

factor (9), the CSFs showed no significant species specificity. For example, both rat and monkey cell lines produced CSF, which apparently was active on mouse bone marrow cells. There was no organ specificity, either. All the cells of liver, kidney, and spleen origin produced CSF. We did note that RLC-10-P3 and M-P3 cells were much less active than JTC-21-P3, JTC-23-P3 and JTC-25-P3 cells, although all these cell lines were derived from rat liver. The gross amounts of protein accumulated in the medium varied considerably in different cell lines and under different culture conditions, but the amount of CSF produced did not always increase proportionally to the total amount of protein.

The readiness of cells to adapt to serum-free conditions was not reflected directly by their ability to produce CSF. According to Katsuta and Takaoka (6), a period of several months was required for the L-929 cells to adapt to the protein-free milieu, whereas JTC-21 and JTC-25 cells grew rapidly as soon as they were transferred to the synthetic medium. In addition, culturing the cells in a protein-free medium was not a necessary condition for CSF production. The addition of 10% calf serum to the L-P3 cell culture did not abolish CSF production, but rather enhanced it significantly (data not shown).

Molecular properties of the produced CSFs. Of the thirteen cell lines examined, L-P3, JTC-25-P3, RSP-2-P3, and JTC-12-P3 cells were arbitrarily chosen for detailed studies of the properties of their CSFs. Isoelectrofocusing showed that the CSFs of the JTC-25-P3 and JTC-12-P3 cell lines were as strongly acidic as the CSF of L-P3 cells (Fig. 1), suggesting that these CSFs are sialoglycoproteins as the CSFs from various sources (13, 19, 21). However, the CSF of RSP-2-P3 cells was slightly less acidic than the CSFs of the other three cell lines (Panel C of Fig. 1).

Because the presence of sialic acid residues in the molecule has been reported to cause CSF to behave abnormally in gel-filtration chromatography even in the presence

Fig. 1. Isoelectrofocusing of the CSFs of L-P3 cells (Panel A), JTC-25-P3 cells (Panel B), RSP-2-P3 cells (Panel C), and JTC-12-P3 cells (Panel D). CSF activity was determined in duplicate in the presence of material corresponding to 1–10 μ l of each fraction. Horizontal arrows show that these fractions were combined and further processed for gel-filtration chromatography with guanidine HCl. Recovery of CSF activity was about 80% on the average.

of 6 M guanidine HCl (13), all four of the above species of CSF were desialylated with neuraminidase before their molecular weight was estimated by the gel-filtration chromatography method. It was thus revealed that three of the four CSFs resembled mutually; *i.e.*, the CSFs of L-P3, JTC-12-P3 and JTC-25-P3 cells all were eluted at 1.35 void volumes, which corresponded to a molecular weight of 40,000 (Fig. 2). When the L-P3 and JTC-25-P3 CSFs were subjected to gel-filtration chromatography without treatment with neuraminidase, both CSFs were eluted at 1.33 void volumes (molecular weight, 47,000) (not shown). In contrast, the majority of the CSF activity of the RSP-2-P3 cells was eluted at 1.45 void volumes (molecular weight, 20,000) (Panel C of Fig. 2). Clearly, the CSF of RSP-2-P3 cells differs from the other three CSFs in both its molecular weight and its molecular charge.

The molecular weight of L-P3 CSF reported here is not consistent with the molecular weight of L60T CSF reported by others (18, 23), although both the L-P3 and L60T cell lines originally were derived from L cells. This discrepancy may simply be due to differences in the methods used to estimate the molecular weight in the different studies.

Morphology of the cells produced. When cultured marrow cells were fixed and stained *in situ* in agar then examined microscopically, granulocytes could be distinguished from monocytes. Thus, we found that the CSF of spleen cell line RSP-2-P3 differed from the CSFs of the other cell lines not only in its molecular properties but also in the granulocyte/monocyte ratio produced. The RSP-2-P3 CSF mainly produced granulocytic colonies, but the other CSFs mainly produced monocytic colonies. Examination of the gel-filtration fractions on both sides of the peak position gave no

Fig. 2. Gel-filtration chromatography of partially purified CSFs in the presence of 6 M guanidine HCl. Panels A–D correspond to Panels A–D in Fig. 1. Each point represents the average of duplicate assays with 150- μ l portions of the material (each fraction, dialyzed and diluted to 2 ml). Arrows 1–5 indicate the elution positions of blue dextran, bovine serum albumin, egg albumin, chymotrypsinogen, and cytochrome C, respectively. Recovery of CSF activity was about 70% on the average. The top fractions and two bilateral fractions (closed circles) from each chromatography were assayed simultaneously for colony morphology. The percentages of monocytic colonies are shown as the average of 2 dishes. The percentage within each group varied by about one-tenth of the mean value.

indication of separation of the granulopoietic from the monocytopenic factor. The increase in CSF concentration tended to enhance the selective action of both types of CSF (Fig. 2; *e.g.*, Panel A, Fraction No. 53 *versus* Fractions No. 50 & No. 56, or Panel C, Fraction No. 57 *versus* Fractions No. 53 & No. 60). As the report of Horiuchi and Ichikawa (4) that the factors specific for monocytopenia and for granulocytopenia were separable by gel-filtration, our present results suggest that there are two distinct types of granulocyte-macrophage CSF (GM-CSF) which significantly differ in the magnitude of amphibiousness, one producing granulocytic colonies, the other producing monocyte-macrophage ones.

Possibly the 7-day-old culture still contained semi-differentiated progenitor cells. Therefore the magnitude of amphibiousness mentioned above should be studied in detail in cultures of various age.

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