Enhancement by Double-Stranded Polyribonucleotides of Production by Cultured Mouse Peritoneal Macrophages of Differentiation-Stimulating Factor(s) for Mouse Myeloid Leukaemic Cells

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Mouse peritoneal macrophages release a factor(s) that stimulates differentiation of a mouse myeloid leukaemic cell line into mature granulocytes and macrophages. Treatment of the macrophages with the synthetic double-stranded polyribonucleotides poly(I)·poly(C) and poly(A)·poly(U) resulted in enhanced release of the factor into the culture medium. The effect was maximal after treatment with polyribonucleotides for 1 h, and the optimal dose of poly(I)·poly(C) was 50 µg/ml. The single-stranded polyribonucleotides poly(I) and poly(C) at the same concentration were far less effective. The differentiation-stimulating factor was detected not only in the cultured medium but also in the cell lysate. Exposure of macrophages to poly(I)·poly(C) enhanced the total activity of the factor in both the culture medium and the cell lysate. The effect of this compound was blocked by the presence of cycloheximide. These results suggest that double-stranded polyribonucleotides enhance production of the differentiation-stimulating factor by peritoneal macrophages.

Mouse myeloid leukaemic cell line M1 can be induced to differentiate into mature macrophages and granulocytes by a protein factor(s) in a conditioned medium of mouse embryo cells or macrophages (Ichikawa, 1969), serum from mice injected with bacterial endotoxin (Fibach & Sachs, 1974) or a variety of ascitic fluids from mice or rats (Hozumi *et al.*, 1974, 1977). Maeda *et al.* (1977) reported that embryo cells required serum or albumin for the production of the factor(s) in the culture medium. However, we found that the macrophages can produce a differentiation-stimulating factor(s) in the absence of serum in the culture medium (Hozumi *et al.*, 1977). For purification of the factor, the serum-free conditioned medium of the macrophages is a good starting material, because it is free from serum-derived proteins. In this work, we tested the abilities of various compounds to stimulate production of the differentiation-stimulating factor(s) and found that synthetic polyribonucleotides, such as poly(I)·poly(C) and poly(A)·poly(U), markedly enhanced the production of the factor(s).

Materials and Methods

Culture of the mouse myeloid leukaemic cell line

Mouse myeloid leukaemic M1 cells originated from a spontaneous myeloid leukaemia in an SL

strain mouse (Ichikawa, 1969). The clone 34T-22 of this cell line used in the present study was cultured in Eagle's minimal essential fluid medium (Nissui Seiyaku Co., Tokyo, Japan), supplemented with double the usual concentrations of amino acids and vitamins and 10% (v/v) heat-inactivated (56°C for 30 min) bovine calf serum, at 37°C under CO₂/O₂ (1:19).

Culture of macrophages

Adult female ICR mice were used in all experiments. The mice were injected intraperitoneally with 2.5 ml of a solution of 5% starch (E. Merck, Darmstadt, Germany) and 5% (w/w) proteose peptone (Difco, Detroit, MI, U.S.A.). Peritoneal cells were collected 3 days after injection of the solution by washing the peritoneum with 0.02 M-sodium/potassium phosphate buffer, pH 7.0, containing 0.15 M-NaCl. Inocula of one million nucleated cells in the phosphate-buffered saline were seeded into 35 mm plastic dishes and incubated for 1 h at 37°C to allow attachment of macrophages. The dishes were then washed twice with the phosphate-buffered saline to remove non-adherent lymphocytes. Adherent macrophages were cultured in serum-free Eagle's minimal essential medium for various periods at 37°C under CO₂/O₂ (1:19) to produce the conditioned medium.

Assay of activity of the differentiation-stimulating factor

Conditioned medium of the macrophages was collected and the cells were removed with a rubber 'policeman'. Suspensions of the cells in the conditioned medium or in fresh Eagle's medium were sonicated for 3 min at 30 s intervals in a Branson Sonifier Cell Disruptor (model W185). Various concentrations of the conditioned medium or cell lysate were added to cultures of M1 cells, and, after incubation for 2 days, the M1 cells were harvested by centrifugation. Suspensions of the cells (2×10^6 – 4×10^6) in 2 ml of serum-free Eagle's medium containing 0.2% of a suspension of polystyrene latex particles (average diameter $1.099 \mu\text{m}$; Dow Chemical Co., Indianapolis, IN, U.S.A.) were incubated for 4 h at 37°C . Then the cells were vigorously washed 3 times with 0.02M-phosphate buffer, pH 7.0, containing 0.15M-NaCl, and the percentage of phagocytic cells among more than 400 viable cells was calculated by the method of Ichikawa *et al.* (1975). All determinations were done in duplicate samples.

Chemicals

Poly(I)·poly(C) was obtained from Yamasa Shoyu (Choshi, Japan), Sigma Chemical Co. (St. Louis, MO, U.S.A.) and P-L Biochemicals (Milwaukee, U.S.A.). Poly(A)·poly(U), poly(I) and poly(C) were purchased from P-L Biochemicals. Cycloheximide was obtained from Sigma.

Results

Differentiation of M1 cells in conditioned medium of mouse peritoneal macrophages

The morphology of M1 cells growing in ordinary maintenance medium is similar to that of myeloblasts from normal haematopoietic organs: the cells have a large round nucleus and little cytoplasm and they do not show phagocytic activity. However, cells treated with the conditioned medium of mouse peritoneal macrophages for more than 2 days had either a small eccentrically located nucleus and abundant cytoplasm, like normal macrophages, or a ring-shaped or polymorphous nucleus, like granulocytes, as previously reported (Ichikawa, 1969; Hozumi *et al.*, 1977). These cells showed phagocytic activity when incubated with polystyrene latex particles. Untreated M1 cells seeded on agar medium formed compact colonies, but, when conditioned medium of the macrophages was poured on to these colonies, they became diffuse: that is, the cells developed locomotive activity (Hozumi *et al.*, 1977). The frequency of these changes in the cells was proportional to the concentration of conditioned medium of the macrophages.

In the present paper, the yield of the differentiation-stimulating factor is expressed as the activity for induction of phagocytic cells in M1 cells. Fig. 1 shows the induction of phagocytic activity in M1 cells by various concentrations of the conditioned medium; although the percentage of phagocytic cells increased progressively with increase in the concentration of conditioned medium, the relationship was linear over only a limited range.

Stimulation of production of differentiation-stimulating factor by poly(I)·poly(C)

Peritoneal macrophages were obtained from the abdominal cavity of mice 3 days after intraperitoneal injection of 5% starch and proteose peptone solution. Cultures of the macrophages were treated with $100 \mu\text{g}$ of poly(I)·poly(C)/ml in serum-free Eagle's medium for 1 h, and then the polyribonucleotide was washed out by the method used by Sehgal *et al.* (1976) for interferon induction. The cultures were fed with serum-free medium and the activity of the differentiation-stimulating factor released into the medium was assayed by determining induction of phagocytic activity in M1 cells. As shown in Fig. 2,

Fig. 1. Induction of phagocytic cells by various concentrations of conditioned medium of macrophages

M1 cells were seeded at 7×10^5 cells per ml into 35 mm Petri dishes with various concentrations of conditioned medium of mouse peritoneal macrophages cultured for 2 days. After incubation of the M1 cells for 2 days, the percentage of phagocytic cells was determined as described in the Materials and Methods section. The points represent the means of duplicate determinations.

Fig. 2. Activity of differentiation-stimulating factor in conditioned medium of macrophages treated with poly(I)·poly(C) *in vitro*

Mouse peritoneal macrophages were harvested and cultured as described in the Materials and Methods section. The cultures were treated with (○) or without (●) 100 μ g of poly(I)·poly(C)/ml in serum-free Eagle's medium for 1 h. Then the polyribonucleotide was washed out and the cells were incubated in serum-free fresh medium. At the indicated times, the medium was collected and 50% (v/v) conditioned medium was assayed for activity to induce phagocytic activity in M1 cells. The points represent the means of duplicate determinations.

the activity of the differentiation-stimulating factor in the conditioned medium was markedly enhanced by the treatment of poly(I)·poly(C). The stimulating effect of the polyribonucleotide on production of the factor was repeatedly observed, but yield of the factor varied in each experiment.

Next the effect of the time of exposure of macrophages to poly(I)·poly(C) on their production of the differentiation-stimulating factor was examined. Cultures of macrophages were treated with 100 μ g of poly(I)·poly(C)/ml for various times, and then thoroughly washed and incubated in fresh medium for a further 5 h. Then the culture fluid was collected and activity of the differentiation-stimulating factor was assayed. It was repeatedly observed that treatment with polyribonucleotide for 1 h was sufficient to induce the maximal stimulation of production of the factor (results not shown). An incubation period of 1 h was therefore used in subsequent experiments.

To examine the dose-dependency of the effect of poly(I)·poly(C) on production of the factor by macrophages, the macrophages were treated with various concentrations of the polyribonucleotide for 1 h and then release of the activity of the factor into the medium for 5 h was measured. As shown in Fig. 3, production of the factor increased progressively with

Fig. 3. Activity of differentiation-stimulating factor in conditioned medium of macrophages treated with various concentrations of poly(I)·poly(C)

Cultures of macrophages were treated with various concentrations of poly(I)·poly(C) for 1 h. The culture medium was harvested after 5 h and 50% (v/v) conditioned medium was assayed for activity of the differentiation-stimulating factor. The points represent the means of duplicate determinations.

increase in the concentration of poly(I)·poly(C) up to 50 μ g/ml, but significantly decreased at a concentration of 200 μ g/ml.

To ascertain whether the effect of poly(I)·poly(C) was due to enhanced release of the differentiation-stimulating factor from the cells or to stimulation of synthesis of the factor *de novo*, we assayed the activity of the factor in cell lysates. Significant activity was detected in a cell lysate prepared immediately after harvesting the peritoneal macrophages. However, the total activity in the cell lysate plus the cultured medium increased when the cells were incubated *in vitro* and was markedly stimulated by treatment with poly(I)·poly(C) (Table 1). These results suggest that poly(I)·poly(C) did not merely stimulate release of pre-existing or newly synthesized differentiation-stimulating factor from the cells, but actually enhanced production of the factor. This view was supported by the finding that the increased activity on treatment with poly(I)·poly(C) was blocked by cycloheximide (Table 2).

Next the specificity of the effect of polyribonucleotide on production of the differentiation-stimulating factor in macrophages was examined. As shown in Table 1, another double-stranded polyribonucleotide poly(A)·poly(U) was also effective, whereas the single-stranded polyribonucleotides poly(I) and poly(C) did not have significant effects on the production of the differentiation-stimulating factor.

Discussion

In this work we found that the double-stranded polyribonucleotides poly(I)·poly(C) and poly(A)·poly(U), but not the single-stranded RNA species poly(I) and poly(C), stimulated production of a

Table 1. *Effects of various synthetic RNA species on production of differentiation-stimulating factor by macrophages*

Cultures of macrophages were incubated with various synthetic polyribonucleotides at 50 µg/ml for 1 h, and then washed thoroughly and incubated in fresh medium for 5 h. The cells were then collected with the culture fluid (total activity), or the culture fluid was removed, the same volume of fresh medium was added, and the cells were harvested with the fresh medium (intracellular activity). The cell suspension was sonicated as described in the Materials and Methods section and a 50%-diluted preparation was assayed for differentiation-stimulating factor. Values are means of duplicate determinations. Abbreviation: n.m., not measured.

Treatment	Activity (% of phagocytic cells)	
	Intracellular	Total
Control (0 time)	1.8	1.8
Control (5 h)	1.9	4.6
Poly(I)·poly(C)	12.5	32.1
Poly(A)·poly(U)	n.m.	25.0
Poly(I)	n.m.	8.9
Poly(C)	n.m.	6.5

Table 2. *Effect of cycloheximide on production of differentiation-stimulating factor stimulated by treatment with poly(I)·poly(C)*

Cultures of macrophages were treated with 50 µg of poly(I)·poly(C)/ml for 1 h, and then the cells were thoroughly washed and incubated in fresh medium for 5 h. Cycloheximide (10 µg/ml) was present throughout the experiment. The cells were collected with the conditioned medium and sonicated. After removal of the cycloheximide by dialysis, the 50% lysate was assayed for activity of the differentiation-stimulating factor. Values are means of duplicate determinations.

Treatment with poly(I)·poly(C) (50 µg/ml)	Addition of cycloheximide (10 µg/ml)	Activity (% of phagocytic cells)
—	—	2.2
+	—	24.1
+	+	4.5

factor(s) stimulating differentiation of mouse myeloid leukaemic M1 cells by mouse peritoneal macrophages *in vitro*.

We also examined the effect of poly(I)·poly(C) itself on differentiation of M1 cells and found that the polyribonucleotide alone did not induce differentiation of the cells, although it stimulated induction of differentiation of the cells by the conditioned medium of the macrophages (unpublished work). Therefore the question arises whether the findings reported in

this paper are the result of contaminating poly(I)·poly(C) in the conditioned medium that was not washed out after treating the macrophages with the polynucleotide. Repeated washing of the cultures of the macrophages after treatment with poly(I)·poly(C) did not decrease the activity of the differentiation-stimulating factor in the conditioned medium. Furthermore the activity of the factor was not decreased by treating the conditioned medium of macrophages exposed to poly(I)·poly(C) with pancreatic ribonuclease, whereas the same treatment of a mixture of conditioned medium of untreated macrophages and poly(I)·poly(C) resulted in loss of the stimulating effect of the polynucleotide on differentiation of M1 cells (unpublished work). These results show that the increase in the activity of the factor in the conditioned medium of macrophages treated with poly(I)·poly(C) is mainly due to activation of the macrophages by the polyribonucleotide.

It has been reported that peritoneal macrophages from rabbits produced autogenous interferon without treatment with any known inducers (Nagano *et al.*, 1966) and that treatment of mouse macrophages with poly(I)·poly(C) induced the production of interferon (Finkelstein *et al.*, 1968). However, interferon was not detected in our preparations of conditioned medium of macrophages. The reason for this discrepancy is obscure and remains to be examined.

It was also shown that injection of poly(I)·poly(C) into mice enhanced the serum activity of a colony-stimulating factor, which stimulated growth and differentiation of mouse haemopoietic cells (McNeill & Killen, 1971), and that the polynucleotide stimulated release of a colony-stimulating factor from human monocytes (Ruscetti & Chervenick, 1974). Characterization of the differentiation-stimulating factor in the conditioned medium of macrophages and studies on the relation between this factor and the colony-stimulating factor remain to be carried out.

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