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Stimulation of Differentiation of Mouse Myeloid Leukemic Cells and Induction of Interferon in the Cells by Double-stranded Polyribonucleotides[1]

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ABSTRACT

Mouse myeloid leukemic MI cells can be induced to differentiate into mature macrophages and granulocytes by differentiation-stimulating factor (D-factor) in conditioned medium of mouse peritoneal macrophages. Double-stranded RNA's, such as the copolymers of polyinosinic and polycytidylic acids and polyadenylic and polyuridylic acids, could not alone induce differentiation of the cells, but enhanced induction of differentiation by low concentrations of the D-factor and induced a significant amount of interferon. Rabbit antiserum to purified L-cell interferon neutralized the antiviral activity of interferon of MI cells. Simultaneous treatment of MI cells with the anti-interferon serum and copolymer of polyinosine and polycytidylic acids and D-factor abolished the enhancing effect of copolymer of polyinosine and polycytidylic acids on the action of the D-factor. These results suggest that the effect of double-stranded RNA's on induction of differentiation of MI cells is mediated by interferon produced by the cells.

INTRODUCTION

The mouse myeloid leukemic cell line MI can be induced to differentiate into mature macrophages and granulocytes by a protein inducer, D-factor,[2] in conditioned medium of mouse embryo cells or macrophages (1, 7, 8) and in a variety of ascitic fluids from mice or rats (6, 7) or by glucocorticoid hormones (4, 5, 14) and various other compounds (18).

In the preceding paper (20), we showed that poly(I)·poly(C) and poly(A)·poly(U) enhanced production of D-factor by mouse peritoneal macrophages. Then we examined the direct effects of some synthetic RNA's on differentiation of MI cells and found that single-stranded RNA poly(I) and poly(C), but not poly(A) or poly(U), were capable of inducing phagocytosis, locomotive activities, Fc receptors, and lysozyme activity in MI cells and mature macrophages and granulocytes; but double-stranded RNA's, such as poly(I)·poly(C) and poly(A)·poly(U), could not alone induce differentiation of the cells (21).

McNeill (16) reported that double-stranded but not single-stranded RNAs potentiated colony formation of mouse hemopoietic cells in the presence of suboptimal concentrations of colony-stimulating factor. Therefore, we examined the effects of synthetic RNA's on differentiation of MI cells in the presence of D-factor, which is thought to be related to the colony-stimulating factor (1). The present paper shows that double-stranded RNA's markedly potentiate induction of differentiation of MI cells in the presence of D-factor and induce interferon in the cells. The role of interferon induction in the effect of RNA's in stimulating differentiation of the cells is also discussed.

MATERIALS AND METHODS

Cells and Cell Culture. Myeloid leukemic MI cells were originally obtained from a spontaneous myeloid leukemia in an SL mouse (8). Clone 34T-22 cells were sensitive to a D-factor in conditioned medium of mouse peritoneal macrophages (20), while clone R4 cells, isolated by Okabe *et al.* (17), were resistant to this factor. Unless otherwise stated, clone 34T-22 cells were used throughout the present study. The cells were cultured in Eagle's minimal essential medium (Nissui Seiyaku Co., Tokyo, Japan), supplemented with double the usual concentrations of amino acids, vitamins, and 10% (v/v) heat-inactivated (56° for 30 min) bovine calf serum at 37° under 5% CO_2.

Assay of Phagocytic Activity. MI cells were incubated with various RNA's for 2 days. The cells were harvested by centrifugation, suspended in serum-free Eagle's medium containing 0.2% of a suspension of polystyrene latex particles (average diameter, 1.099 μm; Dow Chemical Co., Indianapolis, Ind.), and incubated for 4 hr at 37°. Then the cells were thoroughly washed 3 times with 0.02 M phosphate buffer, pH 7.0, containing 0.15 M NaCl, and the percentage of phagocytic cells among more than 400 viable cells were calculated.

Assay of Interferon. Interferon was assayed by a plaque reduction method using mouse L-929 cells and VSV. Synthetic RNA's at the concentrations used in the present experiments had no detectable direct effect on plaque formation of VSV in L-cells. Plaque assay was standardized with NIH reference standard mouse interferon (Catalog No. G002-904-511); one unit in our system was equal to 8.3 NIH international reference units.

Anti-Interferon Serum. Anti-interferon serum was kindly donated by Dr. Yoshimi Kawade and Dr. Yoko Yamamoto of the Department of Cellular and Molecular Virology, Institute for Virus Research, Kyoto University. Antiserum against L-cell interferon (3.4×10^7 IU/mg protein) purified by Yamamoto and Kawade (23) was prepared in a rabbit as described by Iwakura *et al.* (10). It neutralized 10 IU of L-cell interferon at a dilution of 2000.

Neutralization of interferon was assayed by measuring

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[2] The abbreviations used are: D-factor, differentiation stimulating factor; poly(I)·poly(C), copolymer of polyinosine and polycytidylic acids; poly(A)·poly(U), copolymer of polyadenylic acid polyuridylic acids; poly(I), polyinosinic acid; poly(C), polycytidylic acid; poly(A), polyadenylic acid; poly(U), polyuridylic acid; VSV, vesicular stomatitis virus.

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[^{3}H]uridine incorporation into VSV RNA in L-cells by the method of Kawade *et al.* (12).

Assay of Reverse Transcriptase. MI cells (7×10^5 cells/ml) were treated with various synthetic RNA's for 2 days. The cells and cell debris were precipitated from 20 ml of medium by centrifugation at 10,000 g. The virus particles were precipitated from the clarified medium through a cushion of 20% (v/v) glycerol in 0.01 M Tris-HCl buffer, pH 7.6, containing 0.1 M NaCl and 1 mM EDTA, by centrifugation at 40,000 rpm for 60 min in type RP-50 rotor of a Hitachi ultracentrifuge. The resultant pellets were suspended in 0.1 ml of the Tris buffer described above. The reaction mixture in a total volume of 60 μl contained 40 mM Tris, pH 7.8, 120 mM NaCl, 2 mM manganese acetate, 1 mM dithiothreitol, 0.1% Triton X-100, 2 μg poly(rA)·$d(pT)_{12-18}$, 1.25 μCi of [^{3}H]dTTP (4.4×10^4 cpm/pmol; New England Nuclear, Boston, Mass.), and 10 μl of virus suspension. Reactions were carried out for 60 min at 37°, and then trichloroacetic acid-precipitable radioactivity was determined.

Source of D-Factor. Peritoneal macrophages were obtained from ICR mice as described in the preceding paper (20). Macrophages were cultured in serum-free Eagle's minimal essential medium for 2 days, and then the culture fluid was harvested and used as a source of D-factor.

Chemicals. Poly(I)·poly(C), poly(A)·poly(U), and poly(A) were obtained from Yamasa Shoyu, Choshi, Japan. Poly(I), poly(C), poly(U) and poly(rA)·$d(pT)_{12-18}$ were purchased from P-L Biochemicals, Milwaukee, Wis.

RESULTS

Effects of Synthetic RNA's on Differentiation of MI Cells Induced by Conditioned Medium of Mouse Peritoneal Macrophages. The effect of poly(I)·poly(C) on induction of differentiation of MI cells was examined. MI cells (clone 34T-22) were incubated with various concentrations of poly(I)·poly(C) in the presence or absence of 5% (v/v) conditioned medium of mouse peritoneal macrophages for 2 days, and then phagocytic activity was assayed as an index of differentiation of the cells. As shown in Chart 1, poly(I)·poly(C) alone could not induce phagocytic activity at any concentration tested, and 5% conditioned medium alone also had no significant effect. However, in the presence of conditioned medium, poly(I)·poly(C), 1 μg/ml, markedly stimulated induction of phagocytic activity in the cells. Higher polyribonucleotide concentrations of more than 5 μg/ml were cytotoxic to clone 34T-22 and caused less induction of phagocytic activity. The greatest stimulatory effect of poly(I)·poly(C) on induction of differentiation of MI cells was observed with a suboptimal concentration of conditioned medium for stimulation of differentiation of MI cells, and no effect was seen at the concentration of conditioned medium that had the maximal effect in induction of differentiation (Chart 2).

Morphological changes were examined by staining the cells with May-Grünwald-Giemsa. As shown in a previous paper (7), unstimulated MI cells, like myeloblasts, had a large, round nucleus and a little basophilic cytoplasm. On the other hand, differentiated cells obtained with various inducers had either a small nucleus eccentrically located in the cytoplasm like that of normal macrophages or a ring-shaped or segmented nucleus like that of granulocytes.

No differentiated cells were induced by treatment with poly(I)·poly(C) alone, and only about 5% of the cells differentiated into macrophage-like cells on treatment with 5% conditioned medium of peritoneal macrophages only. However, simultaneous treatment with the conditioned medium and poly(I)·poly(C), 1 μg/ml, induced 30 to 70% of the cells to differentiate into macrophage- or granulocyte-like cells. These findings confirm that induction of phagocytic cells by the conditioned medium plus poly(I)·poly(C) is closely correlated with the appearance of morphologically differentiated cells.

The effect of poly(I)·poly(C) on another clone of MI cells, R4, was also examined. This clone was less sensitive than was clone 34T-22 to D-factor in ascitic fluid from rats and conditioned medium of macrophages. As shown in Chart 3, only slight phagocytic activity was induced by treatment of R4 cells with 50% conditioned medium of macrophages, although this treatment caused marked induction of phagocytosis in clone 34T-22 cells. Furthermore, although poly(I)·poly(C) enhanced induction of phagocytic activity in R4 cells in the presence of conditioned medium, the optimum concentration of the polynucleotide for induction was higher than that for sensitive 34T-22 cells.

Like poly(I)·poly(C), another double-stranded RNA, poly(A)·poly(U) when added alone could not induce phagocytic activity in 34T-22 cells, but it potentiated the action of D-factor in conditioned medium (Chart 4). However, a higher concentration of poly(A)·poly(U) than of poly(I)·poly(C) was required for this enhancing effect.

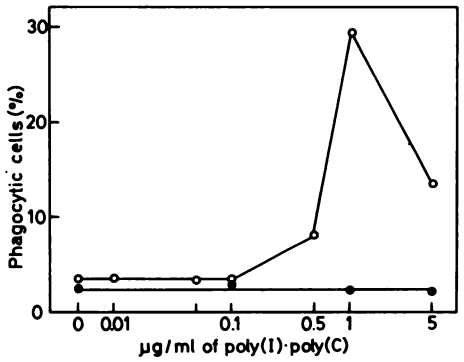

Chart 1. Effect of concentrations of poly(I)·poly(C) on induction of phagocytic activity in MI cells. MI cells were incubated with various concentrations of poly(I)·poly(C) in the presence (○) or absence (●) of 5% conditioned medium of mouse peritoneal macrophages for 2 days, and then their phagocytic activity was assayed as described in "Materials and Methods."

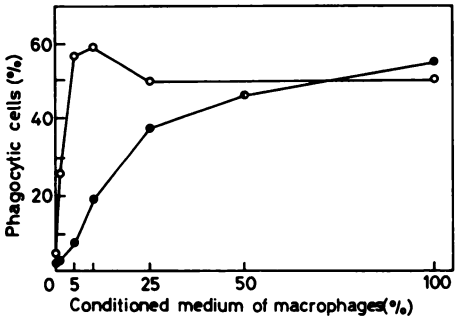

Chart 2. Effect of poly(I)·poly(C) on induction of phagocytic activity in MI cells stimulated with various concentrations of conditioned medium of macrophages. MI cells were incubated with (○) or without (●) poly(I)·poly(C), 1 μg/ml, in the presence of various concentrations of conditioned medium of macrophages for 2 days, and then their phagocytic activity was assayed.

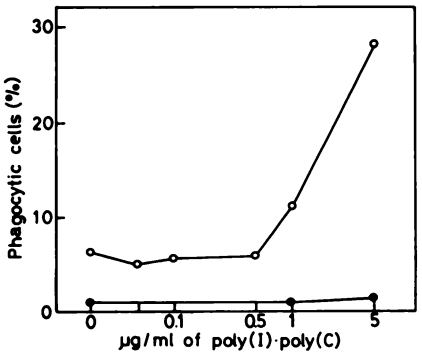

Chart 3. Effect of poly(I)·poly(C) on induction of phagocytic activity in MI cells resistant to D-factor. A resistant clone of MI cells (R4) was incubated with various concentrations of poly(I)·poly(C) in the presence (○) or absence (●) of 50% conditioned medium of macrophages for 2 days.

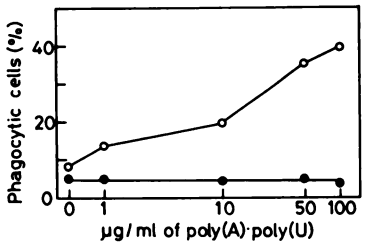

Chart 4. Effect of poly(A)·poly(U) on induction of phagocytic activity in MI cells. MI cells were incubated with various concentrations of poly(A)·poly(U) in the presence (○) or absence (●) of 5% conditioned medium of macrophages for 2 days.

In contrast to double-stranded RNA's, poly(I) or poly(C) alone could induce differentiation of MI cells, as described previously (21). Chart 5 shows the induction of phagocytic activity with poly(I) alone and with poly(I) and conditioned medium of macrophages. The stimulating effects of poly(I) and D-factor in the conditioned medium were synergistic rather than additive. Although poly(C) was less effective than was poly(I), it also caused significant stimulation of induction of differentiation of MI cells (Table 1). Poly(A) and poly(U) had no effect, even in the presence of conditioned medium.

Effect of Poly(I)•Poly(C) on Differentiation of MI Cells Induced by Glucocorticoid Hormone. There may be different cellular mechanisms for induction of differentiation of MI cells by the proteinous D-factor and glucocorticoid hormones, since differentiation of some clones was induced by hormones but not by D-factor (5). Therefore, we next examined the effect of poly(I)·poly(C) on induction of phagocytic activity in the cells stimulated with dexamethasone. Addition of poly(I)·poly(C), 1 μg/ml, had no effect on the action of the hormone (data not shown).

Induction of Antiviral Activity in MI Cells by Synthetic RNA's. Double-stranded RNA's such as poly(I)·poly(C) and poly(A)·poly(U) are known to induce interferon in some cells. Then we examined the relationship between stimulation of differentiation of MI cells and induction of antiviral activity in the cells by synthetic RNA's. It is reported that MI cells release type C RNA virus into the medium (11) and that release of the virus is suppressed by treatment with interferon (13). The activity of reverse transcriptase was assayed to determine release of type C RNA virus from MI cells after treatment of the cells with various RNAs for 2 days. As shown in Table 1, poly(I)·poly(C), 1 μg/ml, poly(A)·poly(U), 100 μg/ml, or poly(I) significantly inhibited the release of the virus; poly(C) slightly inhibited the release; and poly(A) and poly(U) had no effect. These results were confirmed by radioimmunoassay of a protein with a molecular weight of 30,000, a component of type C RNA virus (data not shown). (Purified protein with a molecular weight of 30,000 and the antibody to the protein were kindly supplied by Dr. Kazushige Kai, Institute of Medical Science, Tokyo University.) Next, we measured interferon in the medium. Double-stranded RNA's, poly(I)·poly(C) and poly(A)·poly(U), markedly induced interferon (Table 1). Although slight interferon activity was detected in medium of MI cells treated with poly(I), no activity was induced by other single-stranded RNA's. Therefore, the suppression of release of type C RNA virus may be due to induction of interferon in the cells. Furthermore, stimulation of the differentiation of MI cells by poly(I)·poly(C) or poly(A)·poly(U) in the presence of D-factor seems to be correlated with induction of interferon.

Induction of interferon in MI cells by poly(I)·poly(C) was examined further. The dose dependency of induction of interferon by the polyribonucleotide is shown in Chart 6. Induction of interferon increased with increase in polyribonucleotide concentration up to 2 μg/ml, whereas stimulation of induction of differentiation of MI cells was optimum at a concentration of 1 μg/ml (see Chart 1). The time courses of induction of interferon and stimulation of differentiation by poly(I)·poly(C), 1 μg/ml, are shown in Chart 7. Induction of both interferon and differentiation was apparent after 2 days of treatment of MI cells with

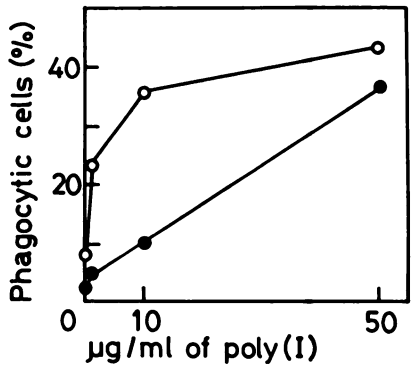

Chart 5. Induction of phagocytic activity in MI cells by poly(I). MI cells were incubated with various concentrations of poly(I) in the presence (○) or absence (●) of 5% conditioned medium of macrophages for 2 days.

Table 1

Induction of differentiation and antiviral activity in MI cells by synthetic RNA's

MI cells were treated with various RNA's for 2 days in the presence or absence of 5% (v/v) conditioned medium of macrophages. Release of type C RNA virus and interferon into the culture medium in the absence of D-factor was determined as described in "Materials and Methods."

RNA's	Concentration (μg/ml)	Phagocytic cells (%) +D-factor	Phagocytic cells (%) −D-factor	Release of RNA virus (%)	Interferon (IU/ml)
None		9.7	2.7	100	ND[b]
Poly(I)·poly(C)	1	41.2	6.4	17	413
Poly(A)·poly(U)	100	39.5	3.5	11	290
Poly(I)	100	62.9	43.9	34	16
Poly(C)	100	19.6	6.2	69	ND
Poly(A)	100	8.1	3.5	109	ND
Poly(U)	100	9.0	3.7	113	ND

[a] Reverse transcriptase activity in the control culture was 7.458 cpm incorporation of [^{3}H]TTP per 10^6 cells per hr.
[b] ND, not detected.

poly(I)·poly(C) and D-factor.

Suppression by Anti-interferon Serum of Stimulating Effect of Poly(I)·Poly(C) on Induction of Differentiation of MI Cells. The effect of anti-interferon serum on stimulation of differentiation of MI cells by double-stranded RNA's was examined to determine the role of interferon induced by double-stranded RNA's. Since the antiserum was prepared against purified L-cell interferon, we first examined its neutralizing activity on MI cell interferon. As shown in Chart 8, the antiviral activities of MI cell interferon and L-cell interferon were completely neutralized by the same concentration of antiserum. Therefore, we next examined the effect of this antiinterferon serum on stimulation of differentiation of MI cells by poly(I)·poly(C). On simultaneous treatment of the cells with antiinterferon serum and poly(I)·poly(C) and D-factor, the stimulating effect of the polyribonucleotide was reduced (Table 2). Since we confirmed that the antiserum did not directly inhibit the action of D-factor and that normal rabbit serum did not suppress the enhancing effect of poly(I)·poly(C), these results indicate that the anti-interferon serum specifically blocked the action of poly(I)·poly(C) by neutralizing interferon induced by the polyribonucleotide. Thus, the results suggest that the enhancing effect of poly(I)·poly(C) on induction of differentiation of MI cells by D-factor is mediated by interferon.

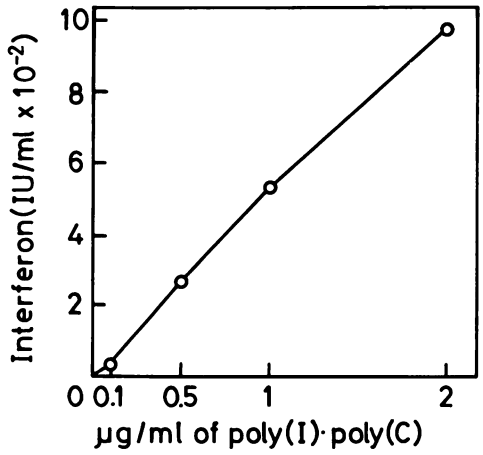

Chart 6. Induction of interferon in MI cells by various concentrations of poly(I)·poly(C). MI cells (7 × 10^5 cells/ml) were treated with various concentrations of poly(I)·poly(C). The culture fluid was harvested after 2 days and assayed for interferon.

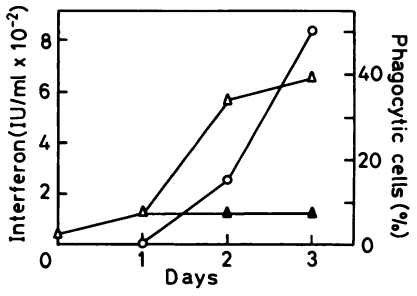

Chart 7. Inductions of interferon and differentiation in MI cells. MI cells were treated with poly(I)·poly(C), 1 μg/ml, and 5% conditioned medium of macrophages for the indicated times, and then interferon (○) in the culture fluid and phagocytic activity (△) of the cells were assayed. Phagocytic activity of cells treated with the conditioned medium only was examined simultaneously (▲).

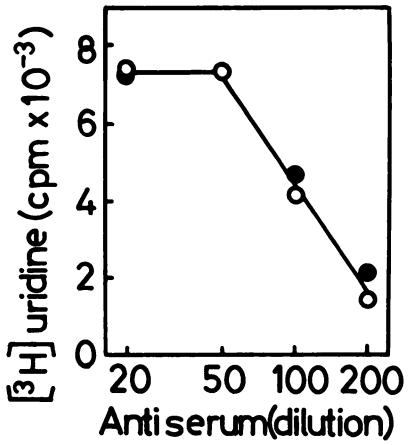

Chart 8. Neutralization of antiviral activity of interferon by anti-interferon serum. Crude interferon (830 IU/0.25 ml) from MI cells (○) induced by poly(I)·poly(C) or L-cells (●) induced by Newcastle disease virus and 0.25 ml of antiinterferon serum diluted as indicated were mixed, and then antiviral activity was assayed by measuring [^{3}H]uridine incorporation into VSV RNA in L-cells. The radioactivity of [^{3}H]uridine incorporated into viral RNA in the absence of interferon and antiserum was 7458 cpm.

Table 2

Effect of anti-interferon serum on induction of differentiation of MI cells by D-factor and poly(I)·poly(C)

MI cells were incubated under various conditions for 2 days, and then their phagocytic activity was assayed. Poly(I)·poly(C) was used at a concentration of 1 μg/ml. Anti-interferon serum and normal serum were diluted 40-fold.

Treatment	Phagocytic cells (%)
None	0.5
D-factor[a] (5%)	3.4
Poly(I)·poly(C)	2.5
D-factor (5%) + poly(I)·poly(C)	31.9
D-factor (5%) + poly(I)·poly(C) + anti-interferon serum	3.7
D-factor (5%) + poly(I)·poly(C) + normal serum[b]	38.5
D-factor (50%)	41.4
D-factor (50%) + anti-interferon serum	39.5

[a] Conditioned medium of macrophages.
[b] Serum from nonimmunized rabbit.

DISCUSSION

In addition to protein inducer, D-factor, various other compounds (18) can induce differentiation of clones of MI cells. Honma *et al.* (2) showed that glucocorticoid hormone stimulated production of D-factor in MI cells and that this factor induced differentiation of the cells. Weiss and Sachs (22) also reported that lipopolysaccharides indirectly induced differentiation of MI cells by inducing D-factor, since the factor was produced before expression of any phenotype of differentiation of the cells.

The mechanism of induction of differentiation of MI cells by poly(I) and poly(C) (Chart 5) are unknown at present, but induction of D-factor may be responsible to the action of the RNA's. However, the stimulating effect of double-stranded RNA's, such as poly(I)·poly(C) and poly(A)·poly(U), on MI cells is not due to the D-factor only because these RNA's alone could not induce differentiation of MI cells. Furthermore, the stimulating effect of RNA's was observed only when a suboptimal concentration of the D-factor was present. The action of RNA's may be related to sensitization of MI cells to the D-factor, since the cells became sensitive to the D-factor when they were incubated with poly(I)·poly(C) alone for 2 days (data not shown). Moreover, it seems likely that polyribonucleotide

may stimulate the action of macromolecular D-factor only, since the polyribonucleotide did not stimulate the action of glucocorticoid.

Synthetic RNA's are reported to have less effect on the induction of interferon in mouse cells than in human or rabbit cells. However, in the present experiments, exposure of mouse MI cells to poly(I)·poly(C), 1 μg/ml, or poly(A)·poly(U), 100 μg/ml, for 2 days resulted in induction of a significant amount of interferon. Furthermore, single-stranded RNA poly(I) also caused slight induction of interferon. This finding is not surprising since it has been shown that some preparations of poly(I) induce interferon in appropriate cells (19). Simultaneous treatment of the cells with antiinterferon serum, poly(I)·poly(C), and D-factor abolished the enhancing effect of the polyribonucleotide on the action of D-factor. These results show that the effect of the polyribonucleotide is mediated by interferon.

We have confirmed that interferon itself stimulated induction of differentiation of MI cells in the presence of D-factor using interferon preparations from MI cells treated with poly(I)·poly(C) and those from L-cells infected with Newcastle disease virus.[3] Recently, Lotem and Sachs (15) reported that interferon enhanced the expression of some genes involved in the differentiation in some clones of MI cells. These findings further support the idea that the enhancing effect of double-stranded RNA's on differentiation of MI cells is at least partly mediated by induced interferon.

On differentiation, MI cells no longer multiply *in vitro* and lose their tumorigenicity in syngeneic mice (3, 9). Therefore, double-stranded RNA's such as poly(I)·poly(C) and poly(A)·poly(U) and interferon may be useful in therapy of these leukemias.

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