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Mechanism of Poly I: Poly C Stimulation of Human Bone Marrow Colony Growth *In Vitro*[1] (40160)

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Bone marrow cells incubated under appropriate conditions *in vitro* give rise to colonies of mature granulocytes and macrophages. These colonies arise from single cells, called colony forming cells or colony forming units in culture (CFU-C) (1–3). The maturation of CFU-C *in vitro* depends on the presence, in the culture medium, of a hormonelike regulator generally termed colony stimulating factor (CSF), (4, 5). The number of colonies developing in culture is dependent on the amount of CSF incubated in the culture (1–3). In humans CSF activity-CSA is produced by mononuclear cells (monocytes and/or lymphocytes - MNC) and tissue macrophages (6–8). Some other substances can enhance colony formation from CFU-C. These substances have no colony stimulating activity of their own. The best studied among these are the synthetic polynucleotides (PolyriboInosinic acid: PolyriboCytidilic acid—Poly I: Poly C, and Poly Adenylic acid: Poly Uridylic acid—Poly A:Poly U) (9–11), endotoxin (11), flagellin (12), and hemolysates of red blood cells (13). The mechanism by which these substances produce their effect has not been fully determined.

In a previous study we domonstrated that Poly I:Poly C added to *in vitro* cultures of human bone marrow cells led to an increase in the size and number of granulocytic colonies produced from CFU-C (10). The present studies were done to determine the mechanism of colony enhancing effect of this synthetic polynucleotide. It has been shown that Poly I: Poly C enhances colony formation only in the presence of mononuclear cells and that addition of Poly I:Poly C to mononuclear cell cultures increases CSA production. These data indicate that the enhancing effect of Poly I:Poly C is the result of stimulation of increased CSA and not through a direct effect on CFU-C.

Materials and methods. Blood and bone marrow used in these experiments were obtained from normal healthy donors in accordance with the rules of the Human Research Committee of the University of Colorado Medical Center.

Blood and bone marrow buffy coats were obtained by gravity sedimentation from specimens collected in heparin. The cells were used unseparated in some experiments and after fractionation in others. Separation was carried out with ficoll–hypaque gradient and glass adherance as detailed below (14, 15).

Bone marrow cultures. The bone marrow was cultured using the technique of Robinson and Pike (3) with certain modifications. Briefly this consisted of petri dishes with two layers of semisolid agar with McCoy's 5A medium and 15% fetal calf serum. The lower layer consisted of a stimulus (feeder) layer which was the source of CSA and the upper layer was the target layer containing CFU-C. The petri dishes were incubated at 37° in an atmosphere of 7.5% CO_2 in air with full humidification. After 12–14 days the colonies obtained from CFU-C's were counted under a dissecting microscope.

Sources of CSF. Whole blood buffy coat feeder layers (1×10^6 cells/ml), which included lymphocytes and monocytes, were used in some experiments as a source of CSA. In other experiments human placental conditioned medium (PCM) was employed. This has been shown to be a potent source of CSA for human bone marrow cultures, and was prepared by the technique of Burgess *et al.* (16).

Conditioned medium obtained by incubation of MNC in McCoy's medium (see below) was also used as a source of CSA and the amount of CSA present in the conditioned

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medium was assayed using separated human bone marrow cells.

Sources of CFU-C. Whole bone marrow buffy coat (unseparated) was used in some experiments while in others bone marrow was used after removal of mature granulocytes and monocytes (separated). Unseparated bone marrow was plated at a concentration of 2×10^5 cells/ml and separated bone marrow was plated at a concentration of 5×10^4 cells/ml.

Cell separation technique (14). Blood or bone marrow buffy coat was diluted 1:1 with McCoy's 5A medium and layered over a ficoll–hypaque gradient, specific gravity 1.077, in a ratio of 6:4. Following centrifugation at 400*g* for 35 min, the cells at the interface were removed and washed twice in McCoy's 5A medium. The interface from blood buffy coat consisted of about 30% monocytes and 70% lymphocytes. This served as the mononuclear (MNC) fraction. The interface from bone marrow consisted of monocytes, lymphocytes and immature white cell precursors and included the CFU-C. The mature granulocytes and nucleated red cells go to the bottom of the gradient. The bone marrow was further processed by glass adherance to remove the monocytes. $0.5–1 \times 10^8$ cells/ml from the ficoll–hypaque gradient were placed in 100 mm glass petri dishes in 10 ml of McCoy's medium for 30 min at 37°. The nonadherant cells were then removed by gentle flushing and suction (15).

Preparation of conditioned media. Mononuclear cells (lymphocytes and monocytes) from peripheral blood were obtained from the interface of the ficoll–hypaque gradient and washed twice in McCoy's 5A medium. The MNC were incubated in different concentrations in McCoy's 5A medium at 37° for 72 hr. Cell concentrations from 0 to 10×10^6/ml were used. Each concentration of cells was incubated with Poly I:Poly C in concentrations varying from 0 to 100 ng/ml. These experiments were carried out with and without 20% autologous serum in the medium. After incubation the medium was centrifuged and the supernatant was assayed, as described above, for its CSF activity. For this purpose 0.1 ml of the conditioned medium was incorporated in the stimulus layer. Poly I:Poly C used in these experiments was obtained from Microbiological Associates, Bethesda, MD in a concentration of 1 mg/ml. (Lot No 90890). Appropriate dilutions were made in physiological saline.

Outline of experiments

a. Culture of unseparated bone marrow with whole blood buffy coat feeder layers as a stimulus with varying concentrations of Poly I:Poly C from 0 to 1.0 μg/ml.

b. Culture of separated bone marrow with PCM as a source of stimulus with varying concentrations of Poly I:Poly C from 0 to 1.0 μg/ml.

c. Assay of conditioned medium, for CSF activity, as obtained with different concentrations of MNC ($0–10 \times 10^6$/ml) and Poly I:Poly C (0–100 ng/ml) in the incubation mixture.

Results. The effect of different concentrations of Poly I:Poly C on the colony forming ability of normal human bone marrow in the presence and absence of mononuclear cells is shown in Tables I and II. When unseparated bone marrow was cultured in the presence of a feeder layer of whole blood buffy coat, there was a marked enhancing effect with the addition of Poly I:Poly C to cultures (Table I). With 0.01 and 0.1 μg of Poly I:Poly C per plate there was a 20% and 45% increase in the number of colonies produced. With a higher concentration of 1.0 μg/plate the increase was still 29% above the control. The difference between control cultures and cultures with a concentration of 0.1 μg is statisically significant ($P < 0.05$). Even though statisically significant changes were not seen at different concentrations there is little doubt that Poly I:Poly C enhances colony growth in the culture system. The changes are quantitativly inconsistent but the general trend is one of enhancement of colony numbers. When the experiment was done with separated human bone marrow, devoid of MNC, with PCM as the source of CSA, the addition of Poly I:Poly C led to a reduction in the number of colonies produced (Table II). These experiments suggested that the effect of Poly I:Poly C was mediated through the MNC which were present in the unseparated bone marrow and/or the feeder layers.

Studies were then done to determine the effect of Poly I:Poly C on CSA production by

TABLE I. EFFECT OF DIFFERENT CONCENTRATIONS OF POLY I:POLY C ON THE NUMBER OF COLONIES PRODUCED FROM 2 × 10^5 UNSEPARATED HUMAN BONE MARROW CELLS STIMULATED WITH A FEEDER LAYER CONTAINING 1 × 10^6 WHOLE PERIPHERAL BLOOD BUFFY COAT CELLS.

	Concentration of Poly I:Poly C μg per plate			
Exp. No.	0	0.01	0.1	1.0
1	155	171 (110)[a]	178 (115)	189 (122)
2	315	366 (116)	529 (168)	432 (137)
3	111	160 (144)	180 (162)	150 (135)
4	35	37 (106)	—	43 (123)
Percent of Control Mean ± SEM	—	120 ± 8.6	145 ± 14.6	129 ± 3.9

[a] Numbers in parentheses indicate percent of control.

TABLE II. EFFECT OF DIFFERENT CONCENTRATIONS OF POLY I:POLY C ON THE NUMBER OF COLONIES PRODUCED FROM 5 × 10^4 SEPARATED HUMAN BONE MARROW CELLS STIMULATED WITH A FEEDER LAYER CONTAINING 0.1 ml OF HUMAN PLACENTAL CONDITIONED MEDIUM.

	Concentration of Poly I:Poly C μg per plate			
Exp. No.	0	0.01	0.1	1.0
1	80	43 (54)[a]	56 (70)	44 (55)
2	80	70 (88)	67 (84)	65 (81)
3	53	59 (111)	42 (79)	36 (68)
4	48	55 (115)	34 (71)	32 (67)
Percent of Control Mean ± SEM	—	91.5 ± 14	76 ± 3.4	68 ± 5.3

[a] Numbers in parentheses indicate percent of control.

MNC in liquid culture. MNC in increasing concentrations (0–10 × 10^6 cells/ml) were incubated in McCoy's medium alone and with Poly I:Poly C in two concentrations (10 and 100 ng/ml). The conditioned medium obtained after 72 hr was assayed for CSA using human bone marrow from which mononuclear cells had been removed. The results of these studies are shown in Fig. 1. It can be seen that the number of colonies stimulated by MNC conditioned medium without Poly I:Poly C was related to the number of MNC. Further, it can be seen that the amount of CSF produced in the MNC conditioned medium for any given cell concentration was enhanced by the addition of Poly I:Poly C. At a cell concentration of 1.25 × 10^6 MNC/ml no CSF activity was detectable in the absence of Poly I:Poly C. With 10 ng/ml of Poly I:Poly C, 38 colonies were stimulated and with 100 ng/ml 12 colonies, indicating increased CSA generation by MNC in the presence of Poly I:Poly C. At cell concentrations of 2.5 and 5.0 × 10^6/ml the control MNC conditioned medium stimulated the production of 16 and 29 colonies, respectively. With the addition of both 10 ng and

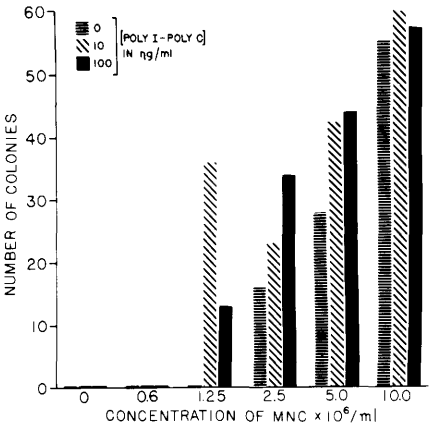

FIG. 1. Colony stimulating factor (CSF) activity from MNC conditioned medium with and without Poly I:Poly C. The different concentrations of MNC employed in the preparation of conditioned medium are shown below the horizontal axis. The vertical axis shows the number of colonies produced when 0.1 ml of conditioned medium was used as a stimulus for 5 × 10^4 separated normal human bone marrow cells. The vertical bars show the number of colonies produced by each type of conditioned medium. The control is shown by horizontal slashed bars, 10 ng/ml of Poly I:Poly C is shown as oblique slashed bars and 100 ng/ml of Poly I:Poly C is shown as solid bars.

100 ng of Poly I:Poly C, CSF production was enhanced. At a MNC concentration of 0.6×10^6/ml and in the absence of MNC no CSF activity was seen with or without Poly I:Poly C indicating that Poly I:Poly C had no CSF activity of its own. With a MNC concentration of 10×10^6/ml the number of colonies produced by conditioned medium from control cultures and from cultures containing Poly I:Poly C was not different. This may have been the maximum measurable response, limited by the number of available CFU-C in this experiment.

Discussion. Previous data from our laboratory (10) and from others (9) has suggested that the enhancing effect of Poly I:Poly C on colony formation was mediated through an action on the CFU-C which is the target cell for CSF. It was suggested that Poly I:Poly C made the target cells more sensitive to CSF. This was based on the observation that showed the enhancing effect of Poly I:Poly C was the same regardless of whether freshly prepared or 7–10 day old feeder layers were used (10). Earlier data from our laboratory have shown that after 5 days whole blood buffy coat feeder layers are not capable of producing additional CSA (17). We interpreted these results to indicate that Poly I:Poly C acted on the target cells. However, in these initial experiments unseparated normal bone marrow cells were empolyed as the target cell layer and the possible effect of Poly I:Poly C on the monocytes in the target bone marrow cells, as well as the MNC in the peripheral blood feeder layers, was not taken into consideration. McNeil *et al.* (9) had also suggested that the effect of Poly I:Poly C was on the target cells and not on CSA production. They had employed protamine sulfate in their culture system. With the addition of protamine sulfate the enhancing effect of Poly I:Poly C was abolished. Protamine sulfate causes cellular uptake of Poly I:Poly C which, it was thought, led to less Poly I: Poly C being available on the cell surface for its action.

The current data, however, suggest an alternative explanation. The enhancing effect of Poly I:Poly C on colony formation was seen only in the presence of MNC with the use of unseparated bone marrow and whole blood buffy coat feeder layers. No enhancement was seen when a fixed amount of CSA was provided in the system in the form of PCM as the stimulus and separated bone marrow cells devoid of MNC as the target layer. Since feeder layers and unseparated bone marrow contained MNC, which are the major source of CSA production, it seemed likely that Poly I:Poly C leads to increased production of CSF from these cells. In the absence of CSF producing cells (PCM with separated bone marrow) the enhancing effect of Poly I:Poly C was not seen.

The experiments using MNC conditioned medium further support this explanation. The addition of Poly I:Poly C to the MNC incubation medium enhanced the amount of CSF produced in the system. This enhancing effect was seen at different numbers of MNC and for different concentrations of Poly I:Poly C. At each concentration of MNC the additon of Poly I:Poly C increased the CSA production proportional to the concentration of Poly I:Poly C in the medium. This effect, however, was not seen when 10×10^6 cells were used in the incubation system.

The possibility that Poly I:Poly C might directly stimulate colony formation has been excluded. In the absence of MNC or with very low concentrations of MNC, Poly I:Poly C had no effect on colony formation. If Poly I:Poly C, transferred from the conditioned medium to the assay system, was responsible for the colony enhancing effect, it should have been seen in incubation mixtures without MNC.

Ruscetti and Chervenick (11) have shown an increase in the CSA release from MNC in the presence of Poly I:Poly C when assayed on separated mouse bone marrow. Our experiments have shown the same effect for human bone marrow and have also shown a regular dose related effect with both the concentrations of MNC and of Poly I:Poly C.

The means by which Poly I:Poly C enhances CSA production by MNC has not been determined. A number of other materials, largely derived from microorganisms, have been shown to have a similar effect (11, 12). Although not clearly demonstrated, it has been suggested that this is the result of antigenic stimulation and this is one possibility to be considered. It is of interest that Poly I:Poly C also stimulates interferon production

by cells (18). The relationship between this and the present noted effect is of particular interest since interferon has been shown to inhibit colony formation in murine and human systems (19–21).

Recent studies have suggested that Poly I:Poly C may be useful in the treatment of various human neoplastic disorders (22). No reports have indicated the effect of this substance on granulopoiesis *in vivo*. The findings reported here indicate that such effects should be sought, and that further studies may give important information regarding the physiologic effects of Poly I:Poly C.

Summary. The effect of Poly I:Poly C on the production of colony stimulating factor activity (CSA) by mononuclear cells (MNC) has been studied. Poly I:Poly C enhanced CSA production from whole blood buffy coat feeders, unseparated bone marrow (containing MNC) and from MNC incubations. Poly I:Poly C had no effect on separated bone marrow (devoid of MNC) stimulated with placental conditioned medium.

These experiments suggest that the colony enhancing effect of Poly I:Poly C is on the production of CSA and not the colony forming unit in culture.

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