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The Effect of Synthetic Double-Stranded Polyribonucleotides on Colony Forming Cells in Normal Human Bone Marrow[1] (38639)

AROOP MANGALIK,[2] WILLIAM A. ROBINSON,[3] AND INDIRA NATH[4]

All India Institute of Medical Sciences, New Delhi 16, India

It has been shown that cells from normal human bone marrow are capable of forming granulocytic colonies in vitro in the presence of an appropriate granulopoietic stimulus (1–3). Under similar conditions blast cells from the peripheral blood and bone marrow of patients with acute granulocytic leukemia (AGL) form granulocytic colonies which are morphologically similar to those of normal bone marrow but are smaller in size (4–7). The reasons for the small colony size are unclear. The possibilities that have been suggested are: (a) A decreased production of granulopoietic substances in AGL (8), (b) a basic defect in granulocyte precursor cells in AGL making them insensitive to the biologic effects of granulopoietic substances (9), or (c) the presence of inhibitory substances preventing granulopoietic factors from acting on these cells (10).

The first suggestion is unlikely in view of our findings of normal levels of granulopoietic substances, as measured by the colony stimulating activity (CSA) of serum and urine in patients with AGL during remission (8, 9). The possibility of inhibitory substances is also not likely in view of our recent work (10) and that of others (11). It has been shown in our own studies that serum and plasma from patients with AGL during the relapse phase of their disease do not inhibit the colony growth of normal human bone marrow cells (10).

It seems reasonable to suggest that the poor colony growth seen with leukemic cells is the result of a basic defect rendering them less responsive to stimulating factors. Addition of cortisone, bacteria, bacterial products, alphamethylglucoparanoside, or trypsinization of cell coats has not produced increased colony growth of either normal or leukemic cells in our laboratory.

Recent studies by McNeill using a double-stranded synthetic polynucleotide, Poly I·Poly C (Poly Inosinic acid, Poly ribocytodilic acid) resulted in enhancement of colony growth in cultures of mouse bone marrow cells (12). The following studies were carried out to determine whether a similar effect might be seen with normal human bone marrow cells. These studies indicate that the addition of Poly I·Poly C to normal human bone marrow cultures enhances the number and size of colonies obtained.

Materials and Methods. The method of tissue culture for colony growth utilized here has been described in detail elsewhere (2, 3). It consists of two layers in semisolid agar, the top layer being a bone marrow target cell layer and the bottom layer being a "feeder layer" of normal human peripheral WBC.

Feeder layer preparation. Peripheral blood is collected from normal human volunteers in heparinized tubes and allowed to sediment by gravity at room temperature from 1 to 2 hrs. The plasma containing the WBC is removed and mixed with a 9:1 concentration of McCoy's 5A medium (with 15% fetal calf serum) and 5% agar in a concentration of 1×10^6 cells/ml. One ml aliquots are then pipetted into 35 mm plastic petri dishes and incubated at 37° prior to use.

Preparation of bone marrow specimens. Aspirate bone marrow specimens collected from the posterior iliac crest of humans without histologic evidence of hematopoietic disorders were used in these experiments. As

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[2, 3] Section of Oncology, Division of Hematology, University of Colorado Medical Center, Denver, Colorado 80220.

[4] Department of Pathology, Royal College of Surgeons, London, England.

with peripheral blood cells, these were collected in heparin, allowed to sediment by gravity at room temperature, and the buffy coat plasma layers separated. The bone marrow cells were mixed with a 9:1 concentration of McCoy's 5A medium (with 15% fetal calf serum) and 3% agar to a final concentration of 200,000 cells/ml. One ml aliquots of this were then plated onto previously prepared feeder layers. Control plates consisted of bone marrow cells plated over cell free agar-medium underlays. Other controls were prepared by mixing Poly I·Poly C into cell free underlays.

After preparation plates were incubated at 37° in a fully humidified incubator with a constant flow of 7.5% CO_2 in air. Colony counts were done at day 15–18 of incubation. Only colonies containing 50 or more cells were scored with a dissecting microscope. For microscopic studies, colonies were removed from the agar with a finely drawn Pasteur pipette and stained with aceto orcein or Giemsa as previously described (2).

Studies with Poly I·Poly C. Poly I·Poly C, supplied by Microbiological Associates, Bethesda, MD, in a concentration of 1000 μg/ml was used in these experiments. Serial tenfold dilutions of Poly I·Poly C were made in sterile saline. Poly I·Poly C concentrations ranged from 0.001–10.0 μg. Parallel experiments were carried out using freshly made feeder layers and feeder layers incubated for 5 days prior to use.

Results. The effect of addition of Poly I·Poly C to normal human bone marrow cultures is depicted in Fig. 1. Shown are the results obtained with four separate normal human bone marrows, each with its own control. The addition of Poly I·Poly C at all concentrations tested from 0.001 to 10.0 μg markedly enhanced colony growth. There was no difference in colony size or numbers when feeder layers were prepared fresh or used after having been incubated at 37° for 5 days. Addition of Poly I·Poly C to bone marrow cultures without feeder layers did not enhance colony growth. In addition to the increase in colony numbers noted there was a dramatic increase (two to three times) in colony size when Poly I·Poly C was added with feeder layers.

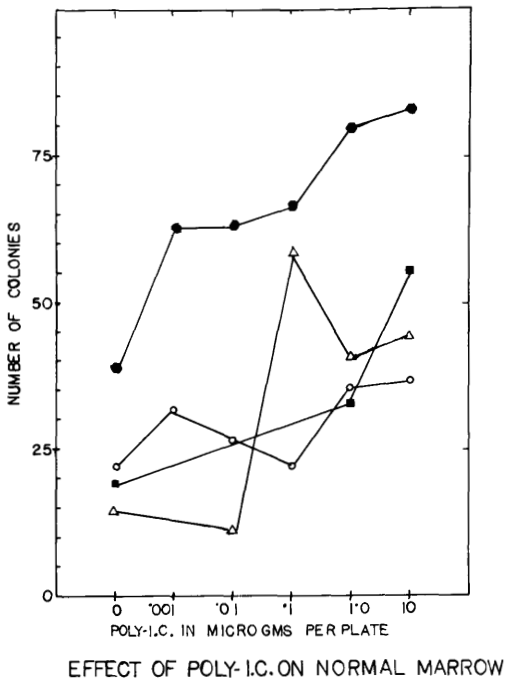

FIG. 1. The effect of Poly I·Poly C on colony formation by normal human bone marrow *in vitro*. Four separate experiments with different bone marrows are shown each with its own control. Concentrations of Poly I·Poly C are shown. The number of colonies formed on plates containing 200,000 nucleated marrow cells stimulated by white blood cell feeder layers is shown. Each point represents the mean colony count of five plates.

Discussion. These experiments with Poly I·Poly C on normal human bone marrow cells show an enhancement of colony growth similar to that noted by McNeill using the mouse system (12).

In our hands this is the first demonstration of a noncellular synthetic material exhibiting enhancement of colony growth of human marrow cells in vitro. The mechanism by which Poly I·Poly C exerts its effect is not known. Previous studies have suggested that the action of CSA on cells is dependent on a cell surface phenomenon which can be inhibited by the action of concanavalin A (13). The data of McNeill et al indicate that the action of Poly I·Poly C is also related to the cell membrane (14). These workers demonstrated that protamine sulfate, which causes intracellular uptake of Poly I·Poly C when

added to cultures of mouse bone marrow, dampens the enhancing effect of Poly I·Poly C on colony growth (14).

The present studies further indicate that the action of Poly I·Poly C is on the target bone marrow cells and not on production of CSA by feeder layer cells. This is evidenced by the fact that colony growth was similar whether fresh feeder layers or feeder layers 5 days old were used as the cell stimulating source. Earlier studies have shown that by the fifth day the production of CSA by feeder layers reaches its maximum and that the majority of cells present are no longer alive or functional (15). It may be concluded that the action of Poly I·Poly C is dependent on the CSA already secreted by the feeder layer cells. Poly I·Poly C alone did not show colony forming activity.

McNeill and Fleming have also shown that injection of Poly I·Poly C into mice results in the appearance of an inhibitor of colony formation in the serum of injected animals (16). The level and timing of appearance of this inhibitor was closely correlated with the appearance of interferon and resembled it closely in physical properties. This is an interesting finding which is being looked for in humans with viral infections using human bone marrow as the target cell.

The enhancement of normal human bone marrow colony growth in vitro by Poly I·Poly Cj has important clinical implications. Cl nical trials of this material in the treatment of various hematologic malignancies are now being carried out. The findings noted here may help to understand the mechanism of action of these materials in clinical situations. These studies also indicate that enhancement of colony growth in vitro can be accomplished using synthetic materials. It is our hope that this can be accomplished with leukemic cells with Poly I·Poly C or other substances in the future. Enhancement of colony growth of the latter may be important and essential in both the understanding and utilization of the knowledge of their responsiveness to cellular regulatory substances.

1. Senn, J. S., McCulloch, E. A., and Till, J. E., Lancet **2,** 597 (1967).
2. Robinson, W. A. and Pike, B. L., *in* "Hematopoietic Cellular Proliferation" (F. Stohlman, Jr., ed.), p. 249, Grune and Stratton, New York (1970).
3. Pike, B. L. and Robinson, W. A., J. Cell. Physiol. **76,** 77 (1970).
4. Iscove, N. N., Senn, J. S., Till, J. E., and McCulloch, E. A., Blood **37,** 1 (1971).
5. Greenberg, P. L., Nichols, W. C., and Schrier, S. L., New Eng. J. Med. **284,** 1225 (1971).
6. Robinson, W. A., Kurnick, J. E., and Pike, B. L., Blood **38,** 500 (1971).
7. Paran, M., Sachs, L., Barak, Y., and Resnitzky, P., Proc. Nat. Acad. Sci. **67,** 1542 (1970).
8. Robinson, W. A. and Pike, B. L., New Engl. J. Med. **282,** 1291 (1970).
9. Robinson, W. A., Entringer, M. A., and Otsuka, A., *in* "The Nature of Leukemia" (P. C. Vincent, ed.), p. 151. V. C. N. Blight Co., New South Wales, Australia (1972).
10. Mangalik, A. and Robinson, W. A., Proc. Soc. Exp. Biol. Med. **141,** 515 (1972).
11. Metcalf, D., Chan, S. H., Gunz, F. W., Vincent, P., and Ravich, R.B.M., Blood **38,** 143 (1971).
12. McNeill, T. A., Immunology **21,** 741 (1971).
13. Otsuka, A. and Robinson, W. A., Proc. Soc. Exp. Biol. Med. **140,** 1441 (1972).
14. McNeill, T. A. and Killen, M., Immunology **21,** 751 (1971).
15. Otsuka, A., Robinson, W. A., and Entringer, M. A., *in* "Proceedings of the Sixth Leucocyte Culture Conference" (M. R. Schwarz, ed.), p. 37. Academic Press, New York (1972).
16. McNeill, T. A. and Fleming, W. A., Immunology **21,** 761 (1971).

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