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The Effect of Synthetic Double-Stranded Polyribonucleotides on Haemopoietic Colony-Forming Cells *in vitro*

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Summary. Addition of low concentrations of the synthetic double-stranded polynucleotides Poly I–Poly C and Poly AU to stimulated cultures of normal mouse bone marrow increased the number of macrophage colonies in the cultures. Double strandedness of the polynucleotides was essential for activity and they were active without the α-globulin which was previously shown to be a necessary co-factor in colony potentiation by some antigens. The evidence suggested that polynucleotides acted at the cell surface to make colony forming cells more responsive to colony stimulating factor.

INTRODUCTION

Whenever cells from haemopoietic tissues are cultured in semi-solid agar a small proportion give rise to colonies which consist of granulocytes, macrophages or a mixture of these cell types. Initiation and growth of colonies is dependent upon a substance known as colony stimulating factor which, for mouse cells, is usually provided by medium conditioned by mouse embryo cultures, certain mouse sera, or preparations from certain human urines. The basic aspects of the culture system have been reviewed by Bradley, Metcalf, Sumner and Stanley (1969). The cells from which colonies arise—colony forming cells (CFC)—are thought to be the progenitor cells of granulocytes and macrophages closely related to the pluripotent haemopoietic stem cells (Worton, McCulloch and Till, 1969; Haskill, McNeill and Moore, 1970).

Study of CFC is of immunological interest for two reasons (i) they are the precursors of cells which can play an important part in antigen processing and (ii) since the stem cells from which they are derived are also the immediate ancestors of lymphoid progenitor cells (McCulloch, 1968) it is of interest to investigate the possibility that these classes of progenitor cells may show similarities in their responses to stimulation.

It was previously shown that injection of a variety of antigens and adjuvants into mice caused an increase in the number of CFC in haemopoietic tissues (McNeill, 1970c). It was also shown that some antigens could potentiate colony formation when added to cultures of normal bone marrow cells *in vitro*, an effect which depended upon combination of antigen with a serum α-macroglobulin (McNeill, 1970a, b).

Synthetic double stranded polyribonucleotides such as Poly I–Poly C and Poly AU have been shown to possess adjuvant action *in vivo* (Braun and Nakano, 1967; Turner, Chan and Chirigos, 1970) and to potentiate transformation of lymphocytes by specific antigen *in vitro* (Friedman, Johnson and Pan, 1969).

In the light of these observations possible effects of these synthetic polynucleotides upon CFC were investigated *in vivo* and *in vitro*. The purpose of this paper is to describe the effects on normal mouse bone marrow cells *in vitro*.

MATERIALS AND METHODS

Reagents

Polyribonucleotides. Most experiments with Poly I–Poly C were performed with a preparation in pyrogen-free isotonic saline obtained from Microbiological Associates, Bethesda. Some experiments were performed with Poly I–Poly C obtained from P. L. Biochemicals Inc., Wisconsin. The homopolymers Poly I and Poly C and the double-stranded Poly AU were obtained from P. L. Biochemicals. These were supplied as a desalted lyophilized product and were reconstituted in sterile isotonic saline prior to use. All preparations were stored at 4° and before each experiment the double-stranded preparations were incubated at 37° for one hour to ensure adequate complexing. Dilutions were made in sterile isotonic saline.

Ribonuclease treatment. A preparation of crystalline bovine pancreatic RNAse was supplied by Dr J. H. Connolly. This was dissolved in 0·01 M phosphate-buffered saline at 40 μg per ml, mixed with an equal volume of Poly I–Poly C (200 μg per ml) and the mixture incubated at 37° for 1 hour.

Protamine sulphate from salmon roe was obtained from L. Light & Co., England. A solution of 200 μg per ml was made in 0·01 M phosphate-buffered saline.

Bacterial endotoxins. A lipopolysaccharide from *Sh. flexneri* was supplied by Dr D. A. R. Simmons. *E. coli* endotoxin 0111:B4 (Difco) was also used, and each of these preparations was reconstituted in sterile distilled water and stored at 4°.

Bone marrow culture

Most experiments were carried out with bone marrow cells from normal 6–12-week-old C57BL mice from an inbred colony maintained in this Department. In some confirmatory experiments cells from CBA and BSVS mice were used.

Marrow plugs from a single femur of two mice were flushed into collecting medium and the cells suspended by pipetting. Collecting medium consisted of Eagle's medium (BHK Eagle's, Burroughs Wellcome, England) supplemented by 10 per cent foetal calf serum, 5 per cent horse serum (Flow Laboratories) and 10 per cent trypticase soy broth (Difco). This medium further supplemented with L-serine (2·5 mg per cent), L-asparagine (1·2 mg per cent), sodium pyruvate (14 mg per cent), DEAE-dextran (6 mg per cent), and with Bacto-agar at 0·3 per cent was used for colony growth.

The Eagle's-agar medium was held at 37° and marrow cells added to give a cell concentration of 5×10^4 per ml. Aliquots of this suspension were transferred to tubes containing a small volume of saline or polynucleotide in saline, mixed and transferred in 1 ml amounts to 35 mm Falcon plastic Petri dishes which contained usually 0·02 or 0·05 ml conditioned medium (Bradley and Sumner, 1968) as the source of colony stimulating factor. In one experiment which will be specifically referred to, normal C57BL mouse serum was used to stimulate colonies. The Eagle's-agar-cell suspension was mixed with stimulant before gelling occurred. In some experiments 30 mm Nunc-sterilin plastic dishes were used. Colony formation in these dishes is poor unless the Eagle's-agar-cell suspension is placed on a 2 ml base layer of the same medium containing 1·2 per cent agar. In any

experiment using this type of dish such a base layer was included, and the conditioned medium incorporated in the base layer. After transfer to the culture dishes the Eagle's-agar-cell suspension quickly gelled and the cultures were then incubated at 37° in a sealed humified box containing 10 per cent CO_2 in air. After seven days incubation colonies were counted at ×16 magnification using an Olympus dissecting microscope.

RESULTS

1. EFFECT OF POLY I–POLY C

Fig. 1 shows the effect of different concentrations of Poly I–Poly C on the number of colonies per culture when conditioned medium was used to stimulate the cultures. Each point is the mean of results from four cultures. This shows that incorporation of Poly I–Poly C resulted in a significant increase in colony number, and that this effect was dependent upon the concentration of polynucleotide, with an optimum at 100 ng/ml. In

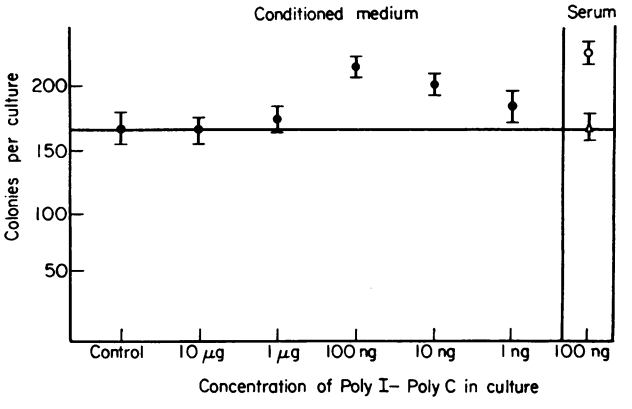

FIG. 1. Effect of Poly I–Poly C on the number of colonies developing in cultures of normal bone marrow. ●, Cultures stimulated with conditioned medium. Cultures stimulated by mouse serum ○, with and △, without 100 ng/ml Poly I–Poly C. Bars are ± SD.

addition it is shown that where mouse serum was used to give the same degree of stimulation of this cell suspension the effect of polynucleotide at 100 ng/ml was the same as that with conditioned medium. Addition of polynucleotide to culture in the absence of colony stimulating factor did not result in any growth of colonies. The dose-response relationship shown was very constant from experiment to experiment and was the same irrespective of the source of Poly I–Poly C, the strain of mouse used as marrow donor, or the type of culture dish used. Although the form of the dose-response relationship was constant its magnitude varied from experiment to experiment, the effect being most marked whenever low levels of baseline colony stimulation were used. This effect is shown in Fig. 2 where it can be seen that Poly I–Poly C increased colony numbers only where the level of baseline stimulation was sub-optimal. No effect was seen when the cultures were maximally stimulated by the conditioned medium.

It is unlikely that the colony enhancement was due to contamination of the Poly I–Poly C preparations with bacterial endotoxin since although endotoxin can potentiate colonies at very low concentrations the α-globulin co-factor is required (McNeill, 1970a).

The results in Table 1 confirm this by showing that in conditioned medium-stimulated cultures, endotoxin from two sources did not result in any increase in colonies.

The specificity of the effect with regard to polynucleotide and to the double-stranded

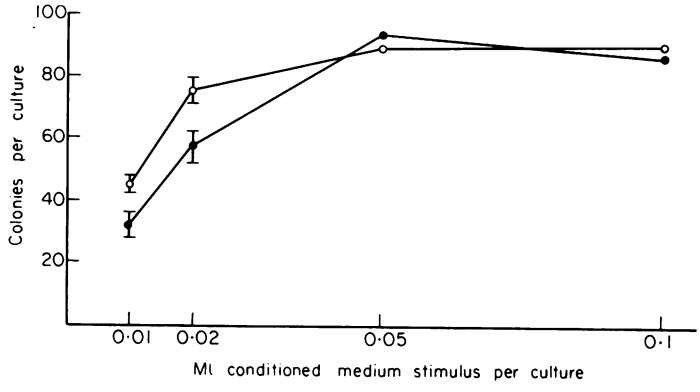

FIG. 2. Colony potentiation by 100 ng/ml Poly I–Poly C. Effect of varying the amount of colony stimulating factor (conditioned medium) in the cultures. ○, Cultures with and ●, without Poly I–Poly C.

TABLE 1

EFFECT OF *E. coli* AND *Sh. flexneri* ENDOTOXIN ON CONDITIONED MEDIUM STIMULATED BONE MARROW COLONIES

Sh. flexneri		*E. coli*	
Concentration in culture	Colonies per culture	Concentration in culture	Colonies per culture
Nil	80	Nil	46
5 μg	8	10 μg	12
500 ng	31	1 μg	30
50 ng	54	100 ng	35
5 ng	71	10 ng	45
500 pg	79	1 ng	51

TABLE 2

EFFECT OF RIBONUCLEASE DIGESTED POLY I–POLY C AND RIBONUCLEASE ON COLONY NUMBERS

Digested Poly I–Poly C		Ribonuclease	
Concentration in culture	Colonies per culture	Concentration in culture	Colonies per culture
Nil	58	Nil	69
1 μg*	54	2 μg	71
100 ng	56	200 ng	72
10 ng	53	20 ng	73
		2 ng	63

* This dilution contained 100 ng/ml ribonuclease.

nature of the material is shown in Table 2 and Fig. 3. Table 2 shows that treatment of Poly I–Poly C with a high concentration of ribonuclease resulted in a loss of the colony enhancing effect and in addition it is shown that the ribonuclease present in the digested

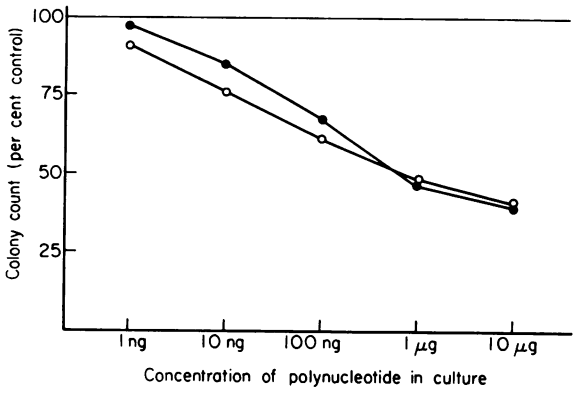

FIG. 3. The effect of single stranded polynucleotides Poly I, and Poly C on cultures of normal bone marrow. ●, Poly I; ○, Poly C.

preparation did not in itself affect colony growth. Fig. 3 shows that the single stranded homopolymers Poly I and Poly C when tested in the same range of concentration as the double-stranded material caused inhibition of colony growth.

2. EFFECT OF POLY AU

Fig. 4 shows the effect of Poly AU compared with Poly I–Poly C in a set of cultures using the same cells under the same cultural conditions. This shows that Poly AU also potentiated

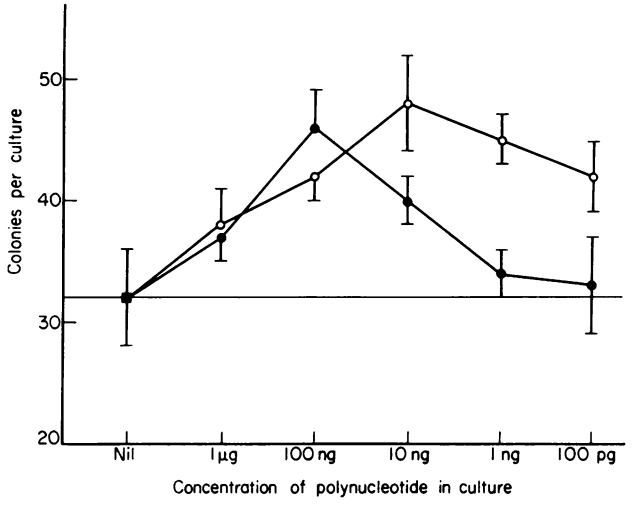

FIG. 4. Comparison of colony potentiation by Poly I–Poly C and Poly AU. Bars represent ±1 SD ●, Poly I–Poly C; ○, Poly AU.

colony development but there was a different dose-response relationship, the optimum effect with Poly AU being obtained at a concentration of 10 ng/ml. This difference was confirmed in several other experiments.

3. POSSIBLE MODES OF ACTION

(a) *Colony stimulating factor production in cultures.* Since it was shown that colony potentiation occurred only in sub-optimally stimulated cultures (Fig. 2) it is possible that the action of polynucleotides may be to stimulate cells in the culture to produce colony stimulating factor. This was investigated by testing agar cultures for colony stimulating activity after several periods of incubation. Three groups of plates were set up, (a) with conditioned medium and Eagle's agar but no cells (control), (b) with conditioned medium, Eagle's agar containing 5×10^4 marrow cells and (c) with conditioned medium, Eagle's agar containing 5×10^4 marrow cells and 100 ng/ml of Poly I–Poly C. The contents of the control (group a) plates were collected after 24 hours incubation at 37° and the contents of four cultures from groups (b) and (c) were collected after 1, 3, 5 and 7 days incubation. Contents of appropriate cultures were pooled, developing colonies dispersed by

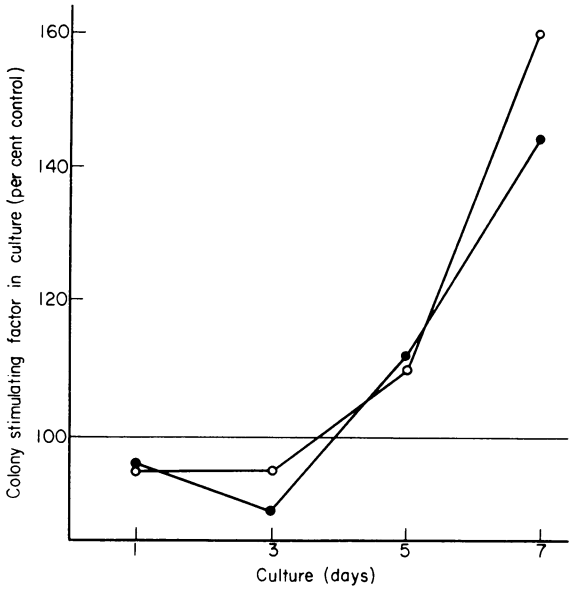

FIG. 5. Colony stimulating activity in pools of bone marrow culture medium during the 7-day culture period. ○, Control cultures; ●, cultures with 100 ng/ml Poly I–Poly C.

pipetting and killed by heating at 45° for 1 hour. Culture pools were stored at −20° and subsequently tested for colony stimulating activity in the same batch of fresh cultures. 0·4 ml of each pool was added to Nunc-sterilin dishes and 2 ml Eagle's 1·2 per cent added to form a base layer on which was placed 1 ml of Eagle's 0·3 per cent agar containing 5×10^4 normal marrow cells. These cultures were incubated for seven days and colonies counted. Fig. 5 shows the colony stimulating activity of the culture pools expressed as a percentage of that of the control pool. This shows that although the amount of colony stimulating factor in the cultures increased after the third day of incubation the addition of Poly I–Poly C did not make any difference to the amount present.

Since an indirect action on CFC through release of colony stimulating factor did not explain the effect of Poly I–Poly C attempts were made to investigate a direct action on the CFC. Such an action could either require uptake of polynucleotide by CFC or could be effective at the cell surface.

(b) *Effect of protamine sulphate.* It is known that the basic protein protamine sulphate increases cellular uptake of infectious viral RNA (Connolly, 1966) and since infectivity is enhanced the protamine sulphate cannot interfere with the biological activity of such RNA. Protamine sulphate has also been shown to increase cellular uptake of Poly I–Poly C (Schafer and Lockart, 1970). Protamine sulphate was added to Eagle's-agar marrow cell suspensions to give a concentration of 2·5 μg per ml before mixing this suspension with different concentrations of Poly I–Poly C. It is evident from Fig. 6 that colony potentiation

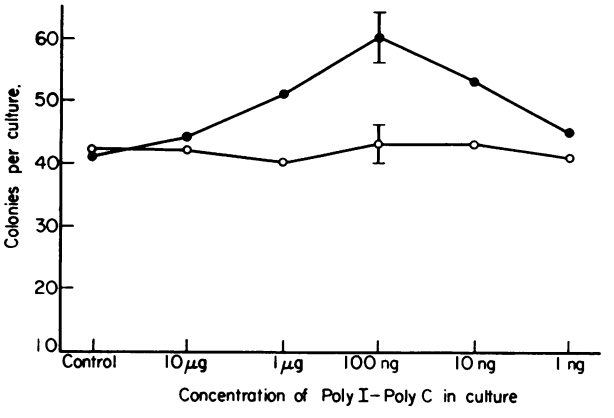

FIG. 6. Effect of protamine sulphate on the colony potentiation effect of Poly I–Poly C. ●, Normal bone marrow cultured with 100 ng/ml Poly I–Poly C; ○, cultured with 100 ng/ml Poly I–Poly C and 2·5 μg per ml protamine sulphate. Bars represent ±1 SD.

by Poly I–Poly C was prevented by incorporation of protamine sulphate since in its absence the same marrow cells with the same stimulus did show the typical dose response effect. In this experiment control cultures were also included which showed that protamine sulphate at this concentration did not in itself alter colony growth. These experiments show that a substance which can increase cellular uptake of Poly I–Poly C prevented colony potentiation and suggested that the effect was the result of some interaction of Poly I– Poly C with the cell surface.

(c) *Pre-treatment experiments.* When normal bone marrow cells were treated in suspension with 100 ng Poly I–Poly C then washed and cultured without polynucleotide the potentiation effect was still obtained. Pre-treatments were carried out at both 37° and 0° for 1 hour, in each case all constitutents of the mixtures were brought to the required temperature before mixing. Table 3 shows that pre-treatment at either temperature resulted in an increase of the colony forming potential of the marrow suspension.

TABLE 3

INCREASE IN COLONY-FORMING POTENTIAL OF NORMAL MARROW CELLS PRE-TREATED FOR 1 HOUR WITH 100 ng/ml POLY I–POLY C

Pre-treatment at 37°	Colonies/10^5 cells
Control	89±8
Poly I–Poly C	136±12
Pre-treatment at 0°	
Control	109±8
Poly I–Poly C	158±6

4. CELL TYPE

Since colonies consist of granulocytes, macrophages or both and since the macrophage element does not appear until the third day of incubation (Metcalf, Bradley and Robinson, 1967; Metcalf, 1969) it is possible to determine whether the effect of Poly I–Poly C is restricted to one or other type of cell by comparing colony counts at intervals during the incubation period in cultures of the same marrow cells either with or without Poly I–Poly C. Table 4 shows such counts at 3, 5 and 7 days of incubation expressed as a percentage of

TABLE 4

EFFECT OF 100 ng/ml POLY I–POLY C ON THE NUMBER OF COLONIES PER CULTURE AFTER 3, 5 AND 7 DAYS OF INCUBATION. EXPRESSED AS A PERCENTAGE OF COLONY NUMBER AT 7 DAYS

	Colony number per cent of day 7 control cultures	
	Control	Poly I–Poly C
Day 3	50	50
Day 5	74	90
Day 7	100	130

the seven day count in cultures without Poly I–Poly C. These are average percentages from two experiments and show that colony development was identical in the two groups of cultures until day 3 (granulocytic stage) but during the macrophage stage the increase due to Poly I–Poly C became apparent.

DISCUSSION

These experiments have shown that the synthetic double-stranded polyribonucleotides Poly I–Poly C and Poly AU when added to stimulated cultures of normal mouse bone marrow cells can increase the number of colonies which develop. Unlike the activity of certain antigens in this respect (McNeill, 1970a, b) polynucleotides did not require a serum α-macroglobulin cofactor to potentiate colony development. It was shown that digestion with ribonuclease destroyed the colony potentiating capacity of Poly I–Poly C and experiments with the single stranded homopolymers Poly I or Poly C showed that double strandedness was essential for activity. Other experiments showed that bacterial endotoxin if present as contaminant in the polynucleotide preparations could not have contributed to the effect.

With regard to possible mechanisms the most obvious is that these substances simply contributed an additional supplement to the culture medium. This is most unlikely since digested Poly I–Poly C and the homopolymers should be as nutritious to developing colonies as the double-stranded complexes yet they had no potentiating effect. Two other possibilities were considered, (i) that the action was to stimulate cells other than CFC to release more colony stimulating factor, and (ii) that the action was on CFC either depending upon uptake of the polynucleotide or through some interaction at the cell surface. It was shown in Fig. 5 that although the level of colony stimulating factor increased

during the later stages of incubation there was no difference between the amount in cultures containing polynucleotide compared with controls.

If cellular uptake was required for activity the addition of protamine sulphate should increase the effectiveness of polynucleotide since it is known to increase the uptake of RNA into cells. The results (Fig. 6) show that the inclusion of protamine sulphate actually abolished the colony potentiation effect of Poly I–Poly C. This suggested that the cell surface was the site of action and support for this was provided by the results of pre-treatment experiments which demonstrated that incubation of cells with Poly I–Poly C for one hour at either 37° or 0° was sufficient to ensure colony potentiation. The most plausible interpretation of these experiments is that Poly I–Poly C acts by increasing the responsiveness of CFC to colony stimulating factor by some action at the cell surface. This interpretation is consistent with the fact that no potentiation was obtained in cultures which were optimally stimulated by colony stimulating factor, and also that Poly I–Poly C had no stimulating effect of colony development in the absence of colony stimulating factor. The effect was obtained only in cultures with suboptimal levels of stimulation (Fig. 2).

It is known that DEAE-dextran can form complexes with polyribonucleotides which may alter their biological properties (Tilles, 1970; Maes and Fernandes, 1970), and since DEAE-dextran is a constituent of the bone marrow culture medium it is possible that such complexes were involved in the observed effects. Attempts to investigate this problem were unsuccessful since colony growth was so poor in the absence of DEAE-dextran.

Although double-stranded polyribonucleotides have a wide spectrum of biological activity the chief interest from an immunological point of view lies in their adjuvant activity (Braun and Nakano, 1967; Woodhour, Friedman, Tytell and Hilleman, 1969; Cantor, Asofsky and Levy, 1970; Turner, Chan and Chirigos, 1970). The number of substances known to possess adjuvant activity is very large and their nature extremely varied. However, it is possible that many of these substances have a common mode of action at the cellular level. It is interesting that those substances which were shown to potentiate bone marrow colonies in association with the α-globulin co-factor (McNeill, 1970a) were either strong immunogens (bacterial flagellin, foreign erythrocytes) or adjuvant active. Double-stranded polyribonucleotides can also potentiate colony formation and do not require the additional α-globulin. We have preliminary results which show that another adjuvant, saponin, can potentiate colonies in very low concentrations (1–100 ng/ml) (Fleming and McNeill, unpublished). All of these results point to a possible association between adjuvant activity and colony potentiation. Such an association raises the problem of defining the connection between the colony forming cell system and immunological responses. Two possibilities, which are not mutually exclusive, can be stated at this stage. First, both systems have one type of cell in common—the macrophage —and the observed effects on each system could be explained by an action on this type of cell. It is interesting to note that the effect of Poly I–Poly C on marrow colonies was confined to the macrophage element of the cultures (Table 4). Further support for this proposition arises from the observations of Friedman, Johnson and Pan (1970) who showed that the effect of Poly AU in potentiating antigen-dependent lymphocyte transformation in cultures of human peripheral blood leucocytes was only observed with mixed cell populations and not with purified lymphocytes.

The second possibility is that since the CFC are very closely related to the precursor cells of immune responses (antigen-sensitive cells) in that each is an early descendant of a common stem cell, they might be expected to show similarities in their responsiveness to

stimulation. The experiments reported in this paper have shown that low concentrations of polyribonucleotides can increase the responsiveness of CFC to colony stimulating factor, and by analogy it is conceivable that they may also increase the responsiveness of antigen-sensitive cells to antigen.

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