cGMP Stimulation of Stem Cell Proliferation

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The process by which resting hemopoietic stem cells become activated is poorly understood, but it has been suggested that cyclic nucleotide levels in the cell may play an important role. In the present study, the effect of various nucleotides and stimulators of nucleotide synthesis upon the formation of in vitro granulocyte colonies has been examined. It was found that physiologic concentrations of 3′,5′-cyclic guanosine monophosphate enhanced the formation of granulocyte-macrophage colonies in the presence of colony-stimulating activity. The cells sensitive to cGMP activation were resistant to thymidine suicide and could not be activated by colony-stimulating activity alone. Therefore it was suggested that the cGMP sensitive stem cell was an ordinarily resting stem cell which was triggered into a proliferative state by cGMP.

GRANULOCYTE PROLIFERATION is thought to derive from echelons of pluripotent stem cells and committed stem cells, which ultimately produce morphologically identifiable granulocytes and macrophages.[1-3] It is believed that, while committed stem cells are actively proliferating, most pluripotent stem cells require activation before they participate in cell production.[4,5] It has been proposed that cyclic nucleotides play an important role in the initiation of the cell reproductive cycle,[6] and the experiments of Byron suggest that both 3′,5′-cyclic adenosine monophosphate (cAMP) and 3′,5′-cyclic guanosine monophosphate (cGMP) can trigger DNA synthesis in pluripotent hemopoietic stem cells.[7] However, the effect of cyclic nucleotides upon granulocyte growth in vitro is unclear. In the present study, we have demonstrated that cGMP stimulates granulocyte growth in vitro. Since the stem cells sensitive to cGMP are resistant to thymidine suicide, they may have been activated from a resting to a proliferating state by cGMP.

MATERIALS AND METHODS

Adult male DBA/2 mice were used in all experiments (Jackson Laboratories, Bar Harbor, Maine). Serum rich in colony-stimulating activity (CSA) was obtained 3 hr after the injection of 25 μg of salmonella endotoxin (Difco Laboratories, Detroit, Mich.). This postendotoxin serum (PES) was pooled and stored at −80°C until used. Concentrated solutions of cyclic nucleotides or other culture additives were prepared in distilled water prior to their use. Control cultures contained added distilled water (10 μliters) equal to the volume of additives. Bone marrow cells were obtained by flushing the hind limb bones of mice with Hanks' balanced salt solution (HBSS), and the cells were counted in triplicate with an electronic counter (Coulter Electronics, Hialeah, Fla.). Agar cultures of bone marrow cells were performed in the manner described by Bradley and Metcalf[2] and Pluznik and Sachs.[8] Marrow cells were mixed with McCoy's 5A medium (M5A) containing 0.3% agar and 15% fetal calf serum, and then 50,000 marrow cells were cultured in 35-mm plastic dishes (Falcon Plastics, Oxnard, Calif.). Cultures were incubated for 7 days in 7% CO_2 at 37°C in a high humidity incubator. Col-

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onies (>50 cells) were counted with the aid of a stereo microscope. Thymidine suicide experiments were carried out in a manner similar to that described by Byron.[7] Marrow cells (5×10^6) were incubated at 37°C for 20 min in 2 ml of HBSS containing 100 μCi ^{3}HTdR (60 Ci/m*M*, New England Nuclear, Boston, Mass.) for a final concentration of 50 μCi ^{3}HTdR/ml. Corresponding control cells were incubated in ^{1}HTdR. ^{3}HTdR uptake was stopped by adding 30 ml of cold HBSS with 15% fetal calf serum and 100 μg/ml ^{1}HTdR. The cells were centrifuged at 200 *g* for 12 min and washed two more times in the HBSS-cold thymidine serum solution. The cells were cultured in the presence of 3% PES, and colony formation by control cells was compared with that of cells preincubated in ^{3}HTdR.

Statistical analysis was carried out by using Student's *t* test to test the null hypothesis. The number of individual cultures was *n* and data were compiled from replicate experiments. When data were normalized as per cent control value, each culture was expressed as the per cent of the mean control value for the control group within that separate experiment.

In order to assess the effect of the various agents to be tested upon the formation of colonies, the appropriate compound was prepared in distilled water and mixed with the medium as the cultures were poured into the dishes. Thus the desired concentration of the additive was established evenly throughout the culture.

RESULTS

Effect of cGMP Upon Colony Formation

In initial experiments, cGMP was added to cultures which did not contain colony-stimulating activity (CSA). In six experiments with a total of 36 cultures containing 10^{-10} to 10^{-6} *M* cGMP, only a rare colony was seen. Thus cGMP did not stimulate colony growth in the absence of CSA. In all other experiments, cultures contained CSA in the form of PES. In data obtained in three experiments, groups of 18 PES-stimulated cultures containing 10^{-10}–10^{-6} cGMP formed more colonies than cultures without cGMP (Fig. 1).

The stimulatory effect of added cGMP was confirmed by experiments in which cultures contained carbamylcholine chloride (carbachol), a stimulator

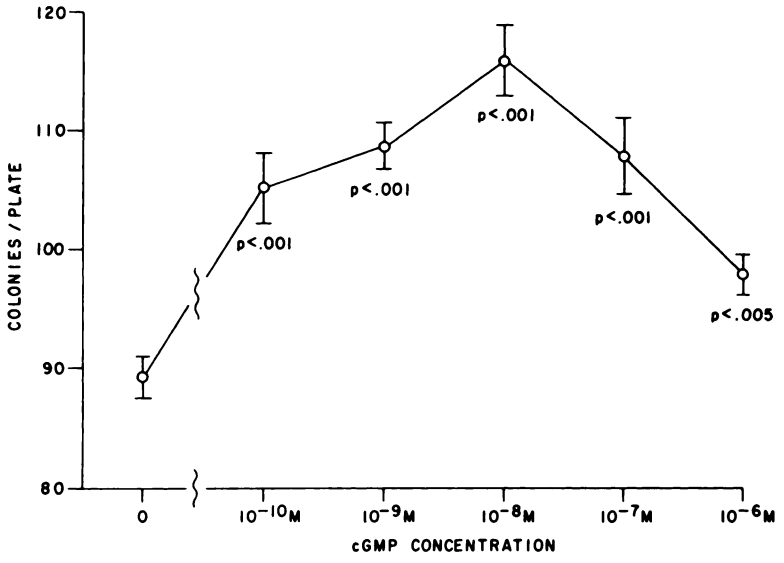

Fig. 1. cGMP stimulation of colony formation. Each culture contained 1% PES. Mean values for 18 cultures at each cGMP concentration are shown. Brackets illustrate the SE.

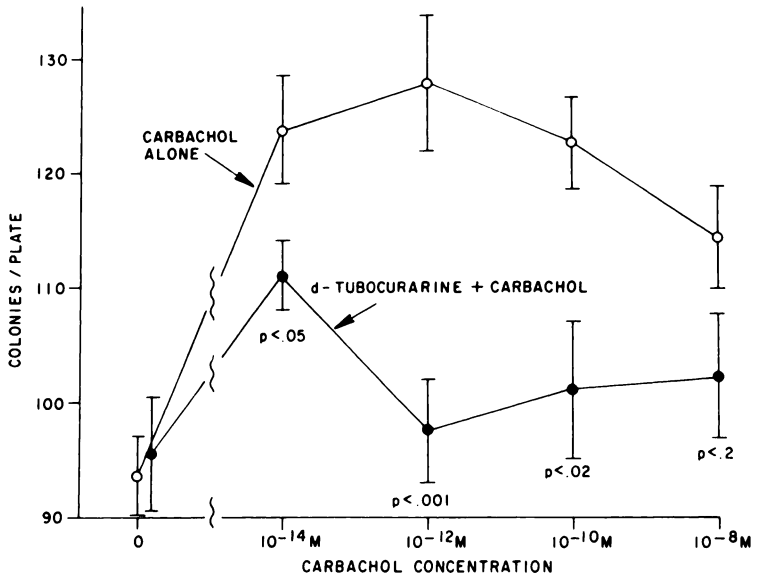

Fig. 2. Effect of agents active at cholinergic receptors upon colony formation. Each culture contained 1% PES. Mean values for groups of 12–16 cultures are shown. Open circles, colony formation in cultures of marrow cells in the presence of carbachol. Closed circles, values for cultures of d-tubocurarine–treated cells in the presence of carbachol and d-tubocurarine. Brackets indicate the SE. The carbachol group differed significantly from the d-tubocurarine group at the 10^{-14}, 10^{-12}, and 10^{-10} M carbachol concentrations.

of intracellular cGMP production. Data were collected from three or four experiments for each agent. Groups of 12–16 cultures containing 10^{-14}–10^{-8} *M* carbachol formed more colonies than cultures without carbachol (Fig. 2, open circles). Since carbachol is believed to act via cholinergic receptors to increase the synthesis of cGMP,[9] we attempted to block the carbachol effect with the anticholinergic agent d-tubocurarine chloride. Marrow cells were preincubated for 5 min with 10^{-6} *M* d-tubocurarine and then incubated in cultures containing 10^{-6} *M* d-tubocurarine and carbachol. Groups of 12–16 carbachol-stimulated cultures of d-tubocurarine-treated cells formed fewer colonies than corresponding cultures not pretreated with d-tubocurarine (Fig. 2). Thus the action of carbachol was inhibited by the cholinergic blocker, d-tubocurarine.

The effect of a variety of other agents upon colony formation was tested in other experiments, and the data from three to five separate experiments are shown in Fig. 3. When 10^{-10}–10^{-6} *M* 5'GMP was added to cultures, no stimulation of colony formation was seen. 10^{-10}–10^{-6} *M* cAMP, or dibutyryl cAMP, also did not significantly affect colony formation. 10^{-12} *M* DL-isoproterenol hydrochloride, a stimulator of adenylate cyclase activity, inhibited colony formation (Fig. 3); 10^{-10} and 10^{-8} *M* isoproterenol did not affect colony growth.

cGMP-sensitive Cell

Further studies were performed in order to compare the characteristics of the cGMP-sensitive cells to those cells which formed colonies under the influence of CSA alone. First, the action of cGMP was observed at various CSA concentrations. Cells were cultured in the presence of concentrations of PES rang-

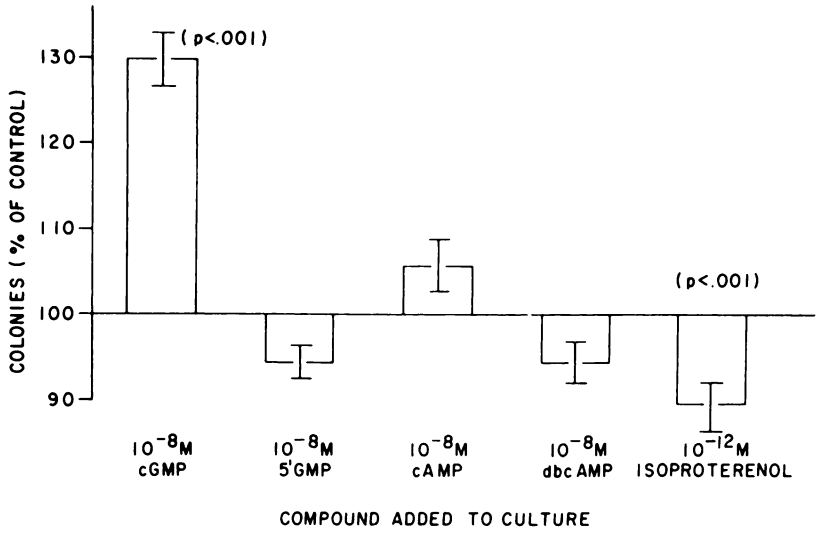

Fig. 3. Effect of cyclic nucleotides and DL-isoproterenol upon colony formation. All cultures contained 1% PES. Bars represent mean values for groups of 12–18 cultures, and the brackets depict the SE. Results normalized for comparison with the mean numbers of colonies formed in control cultures containing only 1% PES. Only the cGMP and isoproterenol groups differed significantly from the control.

ing from 0.5% to 10%, alone and with added 10^{-8} *M* cGMP. The colony formation of groups of 12 cultures from 3 experiments is shown in Fig. 4, and indicates that cGMP stimulation occurred at all tested PES concentrations, including the maximally stimulatory 2% and 5% PES. Thus it was evident that cGMP induced the formation of colonies by a population of cells not ordinarily responsive to CSA.

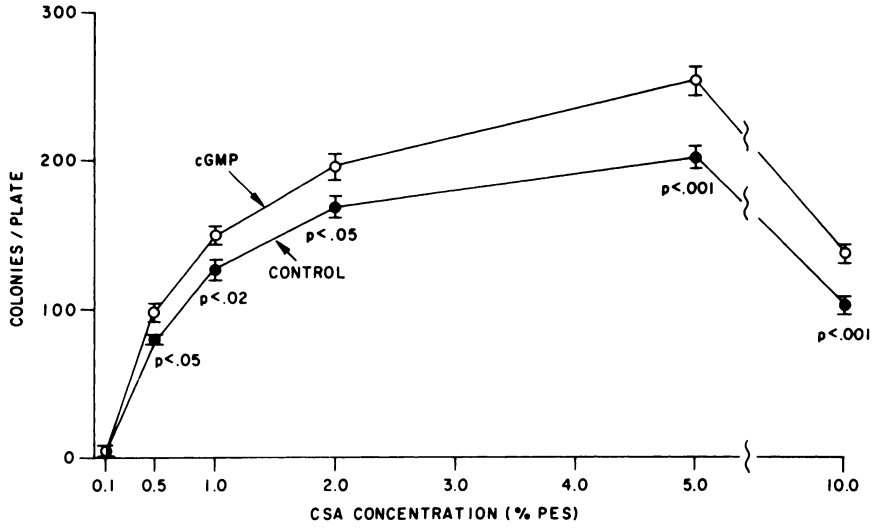

Fig. 4. Effect of cGMP upon colony growth in the presence of various concentrations of CSA. Closed circles, mean values for colony formation in 10–12 cultures containing only postendotoxin serum. Open circles, mean values for 10–12 cultures containing both postendotoxin serum and 10^{-8} *M* cGMP. Brackets indicate the SE.

Table 1. Loss of In Vitro Colony-forming Cells With ^{3}HTdR Pretreatment

Thymidine Treatment	Colonies/Plate With Various Additives (Mean ± SD)		cGMP-sensitive Cells
	3% Postendotoxin Serum	3% Postendotoxin serum + cGMP	
^{1}HTdR	132 ± 18	146 ± 18 ($p < 0.005$)	14
^{3}HTdR	95 ± 14	113 ± 15 ($p < 0.001$)	18

Mean ± SE for groups of 33–35 cultures from 11 experiments. Loss of colony-forming cells responsive to CSA alone (column 2) is 132 − 95 = 37 cells. Loss of colony-forming cells sensitive to cGMP (column 3) is 146 − 113 = 33 cells, a value not significantly different from that obtained in column 2. Thus it can be seen that cGMP-enhanced colony formation (column 2 versus 3) is resistant to ^{3}HTdR treatment and the number of cGMP sensitive cells (column 4) is not reduced by ^{3}HTdR.

Thymidine Suicide Studies

Thymidine suicide studies revealed that the cell cycle characteristics of the cGMP-sensitive cells were different from those of the PES-responsive cell. Cells were preincubated in ^{3}HTdR, allowing cells synthesizing DNA to take up lethal quantities of radioactive thymidine. Then the remaining extracellular ^{3}HTdR was washed away, and the cells were cultured either in the presence of PES alone or PES and cGMP. Cultures of cells treated with ^{3}HTdR could then be compared with controls which had been exposed to ^{1}HTdR. In 11 separate experiments, groups of 33–35 cultures were performed in which the cells had been pretreated with either ^{1}HTdR or ^{3}HTdR. The results, shown in Table 1, demonstrate that the ^{3}HTdR induced loss of in vitro colony-forming cells in the presence of CSA alone (37 colonies per culture) was no different from that in cultures containing both CSA and cGMP (33 colonies per culture), even though cGMP caused an increment in colony formation. The increment induced by cGMP was not reduced by ^{3}HTdR pretreatment, indicating that cGMP-sensitive cells were resistant to ^{3}HTdR killing, and hence were not actively proliferating. In other experiments, the thymidine exposure of the colony-forming cells was increased to 200 μCi/ml in order to kill colony-forming cells which could have been less sensitive to the 50 μCi/ml dose of ^{3}HTdR. In two experiments comprised of 12 cultures for each group, the cGMP-sensitive cells numbered 17/culture after ^{1}HTdR treatment and 15/culture after ^{3}HTdR treatment, indicating that the cGMP-sensitive cells were also resistant to killing with more intense ^{3}HTdR exposure.

DISCUSSION

The present experiments demonstrate that cGMP stimulates colony formation in the presence of colony-stimulating activity. Not only was colony formation stimulated by the addition of cGMP to cultures (Fig. 1), but the same effect occurred if carbachol, a stimulator of cGMP synthesis via guanylate cyclase, was added to the culture medium (Fig. 2). This finding was evidence that an increase in the intracellular content of cGMP enhanced colony formation. This observation was further substantiated by studies in which *d*-tubocurarine, a cholinergic inhibitor, blocked the action of carbachol (Fig. 2). These data suggested that intracellular levels of cGMP may be important for the proliferation of hemopoietic stem cells. Indeed, the present experiments were consistent with the observations of Seifert, who noted that synchronized murine

fibroblasts exhibited increased cGMP levels intracellularly during the initiation of the cell reproductive cycle.[6] Whitfield and Macmanus have also implicated cGMP in the proliferation of thymocytes.[10]

Observations by Byron revealed that both cAMP and cGMP increased the thymidine suicide of pluripotent hemopoietic stem cells, indicating that DNA synthesis had been initiated, but preincubation with the cyclic nucleotides did not increase the number of stem cells which proliferated in vivo.[7] However, it has been difficult to compare the present studies with those of Byron. In Byron's experiments, cells were preincubated for 3 hr in the cyclic nucleotides and then were allowed 9 days to form spleen colonies in vivo. In contrast, in the present studies there was continuous exposure to cyclic nucleotide additives while colonies were formed in vitro.

The inhibition of colony formation by isoproterenol, a stimulator of cAMP synthesis,[11] coincided with the studies of Morley[12] and Tisman,[13] which demonstrated inhibition of granulocyte colony formation in cultures containing prostaglandin E_1, which elevates cAMP.

Cells sensitive to cGMP seem to be a different population from those responsive to CSA alone, because cGMP addition to the culture medium caused an increase in colony formation even at maximal CSA stimulation (Fig. 4), thus indicating that cGMP induced colony formation by a cell not responsive to CSA alone. Further evidence for a distinct cell population sensitive to cGMP was found in the thymidine suicide studies, which indicated that cGMP-sensitive cells had different cell cycle characteristics when compared with those cells responsive to CSA alone (Table 1). The resistance of cGMP sensitive cells to thymidine killing suggested that they were either slowly proliferating or were in the G_0 phase of the cell reproductive cycle. In contrast, 29% of the CSA-responsive cells were killed by thymidine suicide, in keeping with the concept that they were a mitotically active population.[4,5]

The present observations demonstrate the existence of a resting or slowly cycling population of stem cells, which in the presence of cGMP becomes responsive to CSA and forms in vitro colonies. The cGMP-sensitive cell bears a resemblance to the pluripotent stem cell, which also appears to be a slowly cycling or resting stem cell.[5,7] However, the possibility that the cGMP-sensitive cell is an inactive but committed stem cell cannot be excluded. In either case, an increase in intracellular cGMP concentration appears to be important in recruiting resting stem cells into a state of proliferation.

The recent observations of Brown and Adamson indicate that cAMP promotes the growth of dog erythroid colonies in vitro, but cGMP has no effect.[14] Thus it might be postulated that while cAMP is an agonist for erythroid cell growth, cGMP may be the agonist for granulocyte growth. However, it is difficult to compare results obtained in different species of animals, and such a concept will require further testing in a single laboratory animal.

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