Possible Mechanisms of Action of Lithium on Augmentation of in Vitro Spontaneous Myeloid Colony Formation

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ABSTRACT

To understand the possible mechanisms of lithium carbonate-induced neutrophilia, the in vitro effect on human myeloid progenitor cells was examined. A significant increase in spontaneous colony formation (15 of 24 experiments) was observed with the addition of lithium. Increased colony formation seldom occurred when human placental conditioned media as a source of colony-stimulating activity (CSA) was simultaneously added to the cultures. Further data suggest that lithium requires an adherent marrow cell population for this action and that increases in CSA-containing cultures may be due to suboptimal CSA concentrations. Lithium was shown to release CSA from marrow cells and adherent cell population prepared from human bone marrow. Lithium possibly increases spontaneous human myeloid colony development indirectly through CSA release by adherent cells.

INTRODUCTION

Various reports have described consistent elevation of granulocytes accompanying lithium administration in psychiatric patients (12, 14, 18). In 1975, Gupta et al. (8, 9) reported that lithium increased the leukocyte count in patients with Felty's syndrome. They also documented increases in CSA active against murine bone marrow cells in the urine of these patients. Recently, several authors have reported that lithium when administered to patients with various cancers may ameliorate chemotherapy-induced myelosuppression (4, 6, 19, 20, 22) and reduce the duration of granulocytopenic phase induced by chemotherapy in acute myeloid leukemia (5). Lithium has also been used to elevate leukocytes, platelets, and hemoglobin levels in aplastic anemia with some success (1).

Recently, Rothstein et al. (15) have shown that the increase in blood neutrophil count seen after lithium administration is not merely due to demangination but is a result of enlargement of the total blood neutrophil mass and increased neutrophil production. Studies in mice have revealed that lithium enhances the CSA production by lung tissue (9, 10). Using human peripheral leukocyte underlayers, lithium has been shown to increase colony formation in in vitro agar culture system (21). However, thus far, exact mechanism(s) involved have not been clearly elucidated. Herein, we report in vitro experiments performed to delineate these mechanisms in detail using human bone marrow cells.

MATERIALS AND METHODS

These investigations were performed after approval by the local Human Investigations Committee. All patients donating their marrow were informed about the nature of the investigation.

Acquisition of Marrow Specimens. Human marrow specimens were routinely obtained from patients with nonhematological cancers without bone marrow involvement or prior chemotherapy at the time of diagnostic bone marrow aspirations. Marrow was aspirated from the posterior iliac spine. Approximately 1 ml of marrow was placed into a tissue culture tube containing preservative-free heparin (300 units in 2 ml of phosphate-buffered saline).

Marrow Cell Preparation Used in Various Experiments. Buffy coat cells were used for the experiments designed to elicit the enhancement of spontaneous colony formation by lithium and the abrogation of this effect by removal of adherent cells. Buffy coat cells were also used in those experiments showing the effect of lithium on HPCM-stimulated marrow cells. HPCM was prepared as described previously (3).

Ficoll-Hypaque interface cells were used in the experiments designed to show the release of CSA by lithium.

Preparation of Buffy Coat Cells. Marrow specimens were centrifuged at 1200 × g for 10 min in plastic culture tubes (Falcon Plastics, Oxnard, Calif.; Model 3033). The buffy coat was aspirated gently with a Pasteur pipet and subsequently used for various experiments.

Preparation of Light-Density Cells. Marrow specimens were diluted in equal volumes of α-MEM with 15% FCS and centrifuged at 400 × g for 35 min after layering over a cushion of Ficoll-Hypaque (density, 1.077 g/ml) contained in a conical plastic tube (Falcon Plastics; Model 3033) (2). The interface cells were aspirated gently with a Pasteur pipet, washed with phosphate-buffered saline, resuspended in α-MEM, and used for the experiments mentioned later.

Culture Procedure. The culture procedure has been described before except for using HPCM as a source of CSA instead of peripheral blood leukocytes (19). Briefly, the marrow preparations were cultured in equal volumes of double-strength α-MEM with 30% FCS and 0.6% agar (Becto-agar; Difco Laboratories, Detroit, Mich.) giving a final concentration of 0.3% agar with single-strength α-MEM and 15% FCS. For all cultures, 0.1 ml HPCM was used as a source of colony-stimulating factors in underlayers of 0.5% agar and α-MEM with 15% FCS. The same batch of HPCM was used throughout the study. All cultures were plated in triplicate for 7 days in a fully humidified atmosphere of 7% CO₂ and air at 37°C.

Culture Scoring. Cultures were scored on Day 7 using an

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3 The abbreviations used are: CSA, colony-stimulating activity; HPCM, human placental conditioned medium; α-MEM, modified Eagle's medium; FCS, fetal calf serum; CFU-C, human myeloid progenitor cell.

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Olympus dissecting microscope at X25 to 40. They were analyzed for total number of colonies (per plate). The final colony incidence was the mean of the colony incidence from each plate for that particular observation in the study.

Removal of Adherent Cells. Removal of adherent cell population was achieved by incubating 2 × 10⁶uffy coat cells/1 ml of α-MEM and 15% FCS, total volume of 2 ml in 35-mm Falcon Petri dishes (Falcon Plastics; Model 3001) for 3 hr. Nonadherent cells for culture were obtained as described below.

Preparation of Conditioned Media. In the experiments designed to examine the release of CSA, light-density marrow cells were obtained by Ficoll-Hypaque gradient centrifugation (density, 1.077 g/ml) as described above.

To prepare the conditioned media, 2 × 10⁶ interface cells from a Ficoll-Hypaque gradient were incubated per 1 ml of α-MEM and 15% FCS (total volume of 2 ml; total cells 4 × 10⁶). To obtain adherent cell-conditioned media, 2 × 10⁶/ml of interface cells were subjected to adherence procedure as described above (4 × 10⁶ cells/dish), and nonadherent cells were removed by 2 vigorous washings of Petri dishes with α-MEM. Subsequently, the Petri dishes with adherent cells were incubated (each dish containing 2 ml of α-MEM with 15% FCS) for varying periods in a fully humidified atmosphere of 7% CO₂ at 37°. Conditioned media from nonadherent cells were prepared by centrifuging the aspirated nonadherent cells, removing the supernatant, resuspending the cells to a volume of 2 ml in α-MEM and 15% FCS, and reseeding into a new 35-mm plastic Petri dish.

Conditioned media were harvested at specific times and prepared by centrifuging at 2000 rpm for 10 min and passing through a Millipore filter (0.45 μm). Conditioned media were stored at −20° until assayed on 0.5 × 10⁶ nonadherent, light-density (<1.077 g/ml) human marrow cells from a single donor.

Agents Used. Lithium carbonate (Lot 763970; Fisher Scientific Co., Pittsburgh, Pa.) was dissolved in α-MEM in a stock solution of lithium (1000 mEq/liter). A lithium concentration of 1 mEq/liter was used in those experiments designed to examine spontaneous colony formation and the effect of spontaneous colony formation after removal of adherent cells. This concentration is equivalent to serum lithium concentrations achieved in humans with p.o. lithium carbonate.

Lithium carbonate was used at concentrations of 0.5 to 4.0 mEq/liter in experiments examining CSA release. Endotoxin (Escherichia coli 055:B5; Difco Laboratories) was used at concentrations of 100 μg/ml.

Statistics. Differences between the results of experiments with and without lithium were examined by using a Student’s 2-sample t test.

RESULTS

Lithium Effects on Human Marrow Cells: with and without HPCM. To explore the effect of lithium on in vitro culture growth of human myeloid progenitor cells, lithium was used at a concentration of 1 mEq/liter with varying cell numbers (1 to 7.5 × 10⁶/dish) obtained from buffy coat preparations of human bone marrow. Simultaneous experiments were performed using HPCM alone and HPCM and lithium. A total of 9 experiments of this design showed no definite pattern of stimulation with lithium in the presence of HPCM over that achieved with HPCM alone. Because of this unexpected finding, we next examined the effect of lithium on spontaneous colony formation. Twelve experiments were performed at a cell number of 5 × 10⁶/dish, a dose which routinely induces spontaneous colony formation; 10 incorporated lithium in culture plates with and without HPCM, and 2 examined spontaneous colony formation alone. In other experiments, we also examined the effect of lithium at other cell doses on usually both stimulated and unstimulated colony formation. A total of 2 experiments was performed with a cell dose of 1 × 10⁵/dish, 9 experiments were performed with a cell dose of 2.5 × 10⁶/dish, and because of the limitation of cell numbers, 5 experiments were performed with 7.5 × 10⁵ cells/dish.

Chart 1A and B, are representative of experiments examining the effect of lithium on both spontaneous colony formation and that induced with HPCM. As can be seen from these graphs, lithium induced variable enhancement of spontaneous colony formation. For example, the experiment represented by Chart 1A, shows enhancement of spontaneous colony formation at higher target cell numbers (5 and 7.5 × 10⁶ cells), p < 0.05. This difference in spontaneous colony formation induced by lithium, however, was abrogated when HPCM was added to the culture dishes.

Chart 2A shows the cumulative data of the experiments performed with HPCM and lithium. As can be seen from this chart, the effect of lithium on CSA-stimulated plates is variable. Chart 2B is the cumulative data from experiments in which the effect of lithium on spontaneous colony formation was examined. Enhancement of spontaneous colony formation was a common phenomenon. In 6 of 9 experiments at 2.5 × 10⁵, 7 of 12 at 5 × 10⁶, and 2 of 5 at 7.5 × 10⁵ cells, lithium increased spontaneous cloning efficiency by 50% or more (58% of the experiments). Moreover, some of these increases were by greater than 100% (Chart 2B). In 2 experiments, the
variable number of cells per dish. The results are expressed as the percentage of change in CFU-C with lithium compared to that of cultures without lithium. Points at any cell dose, different marrow specimen. In most experiments containing HPCM, the change is minimal, and some even show a decrease of CFU-C. However, without HPCM lithium usually causes a significant increase in spontaneous colony formation.

increase was 13,900 and 800%. However, in only 2 of 11 experiments at 1 x 10⁵, 5 of 15 at 2.5 x 10⁵, 4 of 19 at 5.0 x 10⁵, and 0 of 5 at 7.5 x 10⁵ cells, addition of lithium to the cultures containing HPCM induced an increase of greater than 50% cloning efficiency (22% of the experiments; Chart 2A). Some experiments with both lithium and HPCM show a decrease of CFU-C’s.

The Effect of Adherent Cell Removal on Spontaneous Colony by Lithium. To determine if the lithium effect may be mediated through an adherent cell population in the marrow and not direct stimulation of CFU-C, adherent cell removal was performed in another 3 experiments. Cells were cultured at cell doses ranging from 2.5 to 7.5 x 10⁵/dish.

Chart 3 is representative of these experiments. In all instances, the lithium enhancement of spontaneous colony formation is markedly reduced by the removal of adherent cells. Therefore, it does appear that lithium requires adherent cells for enhancement of spontaneous colony formation.

Lithium Effect on Human Marrow Colony Formation Stimulated with Varying Concentrations of HPCM and Its Modulation by Removal of Adherent Cells. To determine if the variable effect of lithium on colony formation in HPCM-containing cultures may be due to suboptimal amounts of HPCM, lithium (1 mEq/liter) was added to cultures containing 0.025 to 0.3 ml of HPCM. Colony formation was significantly increased in lithium-containing cultures at HPCM concentrations of 0.025 ml (p < 0.05) and 0.05 ml (p < 0.025) as shown in Chart 4A. This enhancing effect of lithium was abolished by prior removal of adherent cells (Chart 4B).

Lithium Release of CSA from Light-Density Human Marrow Cells. To determine if this action is through CSA release, lithium at varying concentrations (0.5, 1.5, and 3 mEq/liter) was incubated with bone marrow and compared to that released by 100 μm g/ml of endotoxin and cells alone. The release of CSA from both bone marrow cell suspensions from which the nonadherent cells had not been removed and adherent cells alone were examined at 24, 72, 96 hr, and 1 week.

Chart 5 is representative of 3 such experiments performed. Lithium did enhance release of CSA from both bone marrow-adherent cells (Chart 5B) and bone marrow cell populations from which nonadherent cells were not removed (Chart 5A). The maximal release in this experiment was at 96 hr of incubation, and after this time, there was a decrease in the activity. CSA release was greater with higher concentrations of lithium (3 mEq/liter) and from cell populations from which nonadherent cells were not removed (Chart 5A). Low concentrations of lithium (0.5 mEq/liter) were no more active than was CSA activity obtained with incubation of cells alone.

To further determine if CSA release from unfractionated, light-density bone marrow cells was solely contributed to by adherent cells, whole bone marrow was further fractionated into nonadherent and adherent cells. CSA release was then determined in all 3 fractions (unfractionated, nonadherent, and adherent). As shown in Table 1, only at the highest lithium concentration (4 mEq/liter), a marginal CSA activity was detected in the nonadherent fraction despite significant CSA release in response to endotoxin. The nonadherent fraction still had a residual 3% monocytes by morphology and latex phagocytosis. However, much higher levels of CSA (23 to 46-fold) were released by unfractionated or adherent cells in response to lithium.

DISCUSSION

This manuscript is the first report of lithium-induced enhancement of spontaneous colony formation and lithium-induced CSA release by human marrow cells. Lithium has been described to release CSA from mouse lung (10) and a CSA from human mononuclear cells active against murine bone marrow (11). However, it has been shown that the CSA released from human peripheral blood monocytes and lymphocytes are multiple, and the CSA active on human bone marrow are of different molecular weights and released at different time periods than those active on murine bone marrow (16). Moreover, Morley and Galbraith (13) did not confirm that lithium enhanced CSA release from human peripheral blood mononu-
clear cells. There are no reports that document lithium-induced CSA release by human marrow cell populations or the release of CSA active on human bone marrow. It has been suggested that the colony-stimulating cell population in marrow may be of more significance in vivo than are peripheral mononuclear cells. We, therefore, decided to examine the possibility that lithium may require a bone marrow-adherent cell population for its mechanism of action and that the mechanism of action may be through the release of CSA from that population. When bone marrow adherent cells are removed prior to culture with lithium, there was a marked reduction of both spontaneous colony formation and lithium enhancement of spontaneous colony formation. When lithium was incubated with light-density (<1.077 g/ml), human marrow cell suspensions and adherent cell populations prepared from these suspensions, it was found to release CSA active on human bone marrow. Furthermore, the activity released from nonadherent bone marrow cell suspensions was minimal, approximately 20 to 50 times less than adherent or unfractionated cell suspensions, and even this little activity could be due to incomplete removal of adherent cells.

There have been 2 previous reports (13, 21) of lithium-induced augmentation of human myeloid colony formation either using cultures containing human peripheral blood cells as an underlayer or a human mononuclear cell source of CSA. This could suggest that lithium may have a direct effect on CFU-C as well as an indirect action through release of CSA. When we examined lithium effects in cultures incorporating HPCM, we noticed a variable effect but significant enhancement was unusual. The concentrations of HPCM used in some of our experiments may not have been those necessary to achieve maximal colony numbers. Therefore, it is conceivable that the target cells in those experiments in which lithium enhanced colony formation despite the prestimulation with HPCM may not have been maximally stimulated. We then examined whether this variability could be due to suboptimal stimulation by HPCM. Lithium did in fact only enhance colony formation at low concentrations of HPCM, and this enhancement was abolished by removal of adherent cells. These results
Human light density bone marrow cells (<1.077 g/ml) were allowed to adhere to plastic surfaces for 3 hr. At the end of this period, nonadherent cells were removed from some cultures as described in 'Materials and Methods' and seeded into new Petri dishes. All cultures were restored to a total volume of 2 ml with α-MEM and sera and then lithium carbonate in concentrations from 1 to 4 mEq/liter, endotoxin (100 μg/ml), or no additional reagents were added. Media were harvested after 7 days and measured for CSA activity against 0.5 × 10⁴ light-density, nonadherent bone marrow.

<table>
<thead>
<tr>
<th>Cell fraction</th>
<th>Lithium (1 mEq/liter)</th>
<th>Lithium (2.0 mEq/liter)</th>
<th>Lithium (4 mEq/liter)</th>
<th>Endotoxin (100 μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unfractionated</td>
<td>0 1.8 ± 3.0</td>
<td>105 ± 4</td>
<td>125 ± 5</td>
<td>79 ± 5</td>
</tr>
<tr>
<td>Nonadherent</td>
<td>0 0</td>
<td>0</td>
<td>3 ± 0.6</td>
<td>77 ± 14</td>
</tr>
<tr>
<td>Adherent</td>
<td>0 87 ± 8</td>
<td>89 ± 12</td>
<td>69 ± 10</td>
<td>83 ± 19</td>
</tr>
</tbody>
</table>

*Colonies, >40 cells.
*Mean ± S.D.

suggest that the variability observed in previous experiments (HPCM plus lithium) might have been due to inadequate HPCM concentrations used inadvertently. Previous publications of appearance of this effect by removal of adherent cells suggests have been due to a similar mechanism, furthermore, the dis
cells may further suggest that the mechanism of indirect action
CSA release from adherent cells and not from nonadherent
cells may further suggest that the mechanism of indirect action
may be due to CSA release from adherent marrow cells. Despite
these findings, one should not conclude that CSA release from an adherent marrow cell population is the only
possible mechanism of lithium action. Also, the variable ratio of
CSA release from unfractionated and adherent cell fractions
from individual marrow specimens attest to the complexity of
CSA release by lithium and could be due to variable blood
contamination of marrow specimens or complex cell-to-cell
interactions. Further experiments are obviously indicated to
more clearly dissect the cellular requirements for lithium-in-
duced CSA release from whole-marrow and adherent marrow
cell populations.

Another interesting facet in these studies is the variable
instances of spontaneous colony formation from human bone
marrow which is not directly related to the CFU-C incidence. It
is possible that further analysis of the mechanism of action of
lithium on in vitro granulopoiesis may identify patients who may
not respond to lithium administration p.o. Further investigations
are under way to examine the effects of lithium on human
marrow which has been previously exposed to chemotherapy
to determine if these changes persist after the administration of
cytotoxic therapy.

Table 1
Lithium-induced CSA release from human bone marrow
unfractionated, nonadherent, and adherent cell fractions

Lithium-Mechanism of Action on Myeloid Colony Formation

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