Enhancement of Colony-stimulating Activity Production by Lithium

By W. G. Harker, G. Rothstein, D. Clarkson, J. W. Athens, and J. I. Macfarlane

Since lithium causes granulocytosis in some patients, its effect upon granulocyte production was investigated using mouse marrow in the agar culture system. When lithium was added to semisolid cultures of mouse marrow, there was no stimulation of colony formation in the absence of colony-stimulating activity (CSA). In addition, lithium did not potentiate the action of already formed CSA. However, lithium did stimulate the production of CSA by lung tissue. Lithium enhancement of CSA production was blocked by puromycin, indicating that lithium action required active new protein synthesis. It was concluded that lithium promoted enhanced granulocyte production in vitro by stimulating the synthesis of CSA.

LITHIUM SALTS have been used in the treatment of arthritis and as salt substitutes for patients needing sodium restriction. However, currently they are being widely used as therapy for manic states. As early as 1950, an increase in the blood leukocyte concentration was noted in patients treated with lithium, and in 1952, it was recognized that the leukocytosis was frequently due to neutrophilia. Subsequent studies have confirmed and expanded the evidence that lithium administration causes neutrophilia, and it has been proposed that lithium may be of use in increasing the neutrophil concentrations of patients with neutropenia.

The mechanism by which lithium produces neutrophilia is not yet known, but the studies of Tisman and Herbert have demonstrated that lithium enhances the production of granulocytes in vitro. In the present experiments, we have investigated the mechanism of this action of lithium. It has been found that lithium stimulates granulocyte colony formation in agar plates; this action is mediated by enhancement of colony-stimulating activity (CSA) production.

MATERIALS AND METHODS

Adult male CBA mice were used in all experiments. Bone marrow was obtained by flushing the hind-limb bones with McCoy’s 5A medium (M5A) with 15% fetal calf serum (FCS). Clumps of cells were eliminated by aspirating repeatedly through a 25-gauge needle. The cell concentrations of the marrow suspensions were measured electronically using a Coulter Counter and the means of triplicate counts were determined. The cell concentrations were adjusted with M5A and 50,000 marrow cells were then cultured in individual 35-mm plastic dishes in a manner similar to that previously described. All cultures consisted of a lower and upper layer, each with a volume of 1 ml. The lower layer was composed of M5A with 15% FCS and 0.5% agar. The upper layer consisted of M5A with 15% FCS and 0.3% agar. After a 7-day incubation at 37°C, 7% CO₂ in a high humidity incubator, the colonies (greater than 50 cells) were scored with the aid of a stereomicroscope. LiCl was dissolved in distilled water in appropri-
ate concentrations for use in the cultures and 0.05-ml volumes were added to each of the upper and lower layers to insure even distribution of lithium throughout the culture. With the lithium concentrations employed, the pH of M5A was not altered. Serum rich in CSA was generated by injecting mice with 25 \( \mu \)g of *Salmonella* endotoxin and collecting the serum 3 hr later. The post-endotoxin serum (PES) was pooled and stored at \(-80^\circ\)C until used. When PES was added to cultures, the appropriate volume of PES to yield a final desired concentration throughout the culture was added to the lower layer before the upper layer was poured. For example, the addition of 0.1 ml PES to the lower layer of a culture whose final volume was 2 ml resulted in a culture containing 5\% PES.

CSA produced by mouse lungs was obtained by the method of Sheridan and Metcalf.\textsuperscript{11} For each 40 mg of lung, 1 ml of serum-free M5A was used. Lung conditioned medium (LCM) was collected after 48 hr of incubation and was heated to 56\(^\circ\)C for 30 min before it was centrifuged at 12,000 \( g \) for 15 min at 4\(^\circ\)C. Then the LCM was dialyzed against distilled water containing 200,000 units/liter penicillin and 200 mg/liter streptomycin. The dialyzed LCM was centrifuged at 4\(^\circ\)C for 15 min at 12,000 \( g \). Finally, LCM was assayed for CSA by adding 0.1-ml aliquots to the underlayers of marrow cultures which did not contain lithium.

**RESULTS**

The effect of lithium upon granulocyte growth in vitro can be evaluated by testing the effect of lithium upon each of the components of the agar culture system: (1) If lithium promotes the growth of mouse granulocytes, it could do so by direct stimulation of the colony forming cell. (2) Another possibility could be that lithium enhances the action of already formed CSA. (3) Finally, lithium might promote increased production of CSA by tissues capable of producing CSA. These possibilities were individually examined in the following experiments.

*Effect of Lithium Upon the Colony-forming Cell*

To ascertain whether lithium had a direct stimulatory effect on granulocyte-macrophage progenitor cells, mouse marrow cells were cultivated in M5A-agar to which no CSA had been supplied but which contained various concentrations of lithium. After the cultivation period, the colonies were counted and the relationship between colony number and lithium concentration examined. In cultures containing lithium at 0.5, 1.0, 3.0, or 3.0 mEq/liter, only a rare colony was seen. Similarly, control cultures without added lithium contained only an occasional colony. Thus lithium alone did not stimulate the colony-forming cells.

*Effect of Lithium Upon the Action of Preformed CSA*

To evaluate whether lithium enhanced the activity of CSA, the effect of lithium concentrations of 0.5, 1.0, 3.0, and 5.0 mEq/liter in cultures containing 1\% PES was examined. Colony numbers in these cultures indicated that lithium did not enhance colony formation and in fact had a slight inhibitory effect at the higher lithium concentrations (Fig. 1). Other experiments revealed that the effect of lithium upon the CSA of mouse lung origin was similar. Cultures containing 5\% LCM and added lithium were compared with control cultures containing LCM only. The mean values for groups of six cultures were: no added lithium, 28 colonies/plate; 0.5 mEq/liter lithium, 25 colonies/plate; 1.0 mEq/liter lithium, 26 colonies/plate; 3.0 mEq/liter lithium, 22 colonies/plate; and 5.0 mEq/liter lithium, 20 colonies/plate. Thus the action of pre-
formed LCM was not enhanced by lithium and slight inhibition of colony growth was observed at the high lithium concentrations.

The Effect of Lithium Upon the Production of CSA

To evaluate whether lithium enhanced CSA production, 4 mg of minced mouse lung was included in the underlayers of culture plates—either control plates without added lithium or cultures to which lithium had been added. When the lower layers had solidified, upper layers containing 50,000 mouse marrow cells were added. After the 7-day culture period, the effect of lithium upon colony formation was observed. The result of these studies is shown in Fig. 2, which demonstrated that lithium addition enhanced colony formation, probably by stimulating increased CSA production by the lung feeder layers.

Other experiments confirmed stimulation of CSA production by lung. Lungs were incubated in media containing either no added lithium or various concentrations of added lithium. Then 0.1-ml aliquots of the LCM were assayed for CSA in agar cultures of 50,000 mouse marrow cells. After the culture period, the number of colonies formed was counted and used as a measure of CSA production. The results, shown in Table 1, represent the means and standard deviations of sextuplicate cultures for each LCM. It can be seen that the incubation of lungs in the presence of lithium increased the production of CSA by the lungs.
Table 1. Effect of Lithium Upon the Ability of Lung to Produce CSA in LCM

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Lithium Concentration (mEq/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>32.5 ± 5.85</td>
</tr>
<tr>
<td>2</td>
<td>12.7 ± 2.1</td>
</tr>
<tr>
<td>3</td>
<td>17.5 ± 3.1</td>
</tr>
<tr>
<td>4</td>
<td>30.8 ± 6.2</td>
</tr>
</tbody>
</table>

Values are CSA expressed as colonies per plate. Means ±SD of groups of six values for cultures of 50,000 mouse marrow cells are shown.

*p = <0.05.

Puromycin Inhibition of Lithium-stimulated CSA Production

Puromycin, a protein-synthesis inhibitor, was used to clarify further the nature of lithium enhancement of CSA production. In this experiment 4–6 individual LCM were generated in the presence of lithium at 0.5, 1.0, 3.0, or 5.0 mEq/liter with puromycin 50 mg/ml added. Then the resultant LCM was dialyzed to remove residual puromycin and lithium before assay for CSA in the agar cultures. It was found that none of the lithium-puromycin-incubated lungs was able to produce measurable CSA. To exclude the possibility that residual puromycin or other inhibitor had prevented colony formation, the CSA of PES alone was compared with the CSA of the PES in the presence of puromycin-incubated LCM. The puromycin LCM did not inhibit the action of CSA derived from PES. Thus it was concluded that the inactivity of lithium-puromycin-incubated LCM was truly due to an absence of CSA.

DISCUSSION

In the present studies we have demonstrated that lithium could enhance the growth of granulocytes in vitro, but this stimulation did not reflect a direct effect of lithium upon the colony-forming cell. Thus, lithium did not promote colony growth in the absence of added CSA nor did it potentiate the effect of CSA added to cultures, either in the form of PES or LCM. In contrast, the addition of lithium to cultures containing lung feeder layers (i.e., living cells capable of making CSA) resulted in increased colony formation. Also, lithium stimulated increased production of CSA by lungs incubated in serum-free medium. These studies established that lithium enhanced colony formation by murine cells by increasing CSA production. Tisman and Herbert have previously shown that lithium promoted the growth of human granulocytes in cultures with feeder layers acting as a source of CSA.7 Although those studies were compatible with lithium stimulation of CSA production, the possibility that lithium might potentiate CSA or directly stimulate human stem cells was not investigated. Thus, although the present study demonstrated lithium stimulation of CSA production by mouse cells, other experiments with human cells will be necessary to determine the site of action of lithium in a human cell system.

It is of interest that lithium-stimulated CSA production is inhibited by the protein-synthesis blocker puromycin. The experiments of Sheridan and Metcalf11 suggest that the effect of puromycin is one of inhibition of protein synthesis rather than of nonspecific toxicity and cell death, because in the presence
of puromycin, another active cell process, incorporation of \(^3\)H-uridine, continues. Thus it appears that new protein synthesis is necessary for lithium in the lung incubation system. It is not yet clear whether new protein synthesis is required for synthesis de novo of CSA or for the synthesis of a protein necessary for secretion of CSA, although those studies suggest that in an adherent peritoneal cell system, early release of CSA is resistant to inhibition by a protein-synthesis blocker. Although the present studies\(^1\) identify an in vitro action of lithium, caution must be exercised in the interpretation of these findings in the intact animal. For example, the separate control of granulocyte production and release into the blood\(^2,3\) suggest that another mechanism is likely to be responsible for lithium-induced neutrophilia. Further studies in vitro and in vivo will be necessary to clarify completely the action of lithium.

REFERENCES

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