The Effect of Lithium on Growth Factor Production in Long-Term Bone Marrow Cultures

By H. Elizabeth McGrath, Chi-Ming Liang, Thomas A. Alberico, and Peter J. Quesenberry

We have previously reported that lithium chloride (LiCl) stimulates the production of granulocyte–macrophage colony-forming cells (GM-CFC), pluripotent stem cells (CFU-S), and differentiated granulocytes, macrophages and megakaryocytes in murine Dexter marrow cultures and that this effect appears to be mediated indirectly by a radiosensitive adherent marrow cell. In this study we have established that exposure of murine Dexter cultures to LiCl (4 meq/L) causes an increase of colony-forming cell megakaryocytes (CFU-meg) over 1 to 6 weeks of culture in both supernatant (188% to 611%) and stromal phases (123% to 246%). Moreover, we have shown that lithium treatment of either irradiated (1,100 rad) or unirradiated stromal cells increased production of activities stimulating formation of megakaryocyte, granulocyte, macrophage, and mixed lineage colonies and proliferation of the factor-dependent cell line, FDC-P1. This FDC-P1 stimulatory activity was completely blocked by an antibody to purified recombinant granulocyte–macrophage colony stimulating factor (rGM-CSF). The baseline or lithium-induced–stromal-derived bone marrow colony stimulating activity was partially blocked by the antibody to rGM-CSF and by an antibody to purified colony stimulating factor I (CSF-1); the two antibodies combined resulted in greater than 90% inhibition of the lithium-induced marrow stimulatory activity. In addition, radioimmunoassay (RIA) showed that although CSF-1 was detectable in supernatants of these cultures, exposure to lithium did not increase CSF-1 levels. These data indicate that Dexter stromal cells produce CSF-1 and GM-CSF and that lithium appears to exert its stimulatory effects on in vitro myelopoiesis by inducing production of GM-CSF.

LEUKOCYTOSIS has been recognized as a side effect of lithium therapy since 1950,1 and the use of lithium therapy for various hematopoietic disorders has been explored.2-3 The stimulatory effect of lithium has been found mostly in the granulocyte lineage, although it appears to act on the megakaryocyte and the erythroblast lineages as well.4-6

The mechanism of action of lithium on hematopoiesis has not been elucidated.6-7 Some reports suggest that lithium acts directly on the stem cells,9-10 but there are data supporting the view that lithium acts indirectly via a secondary effector cell population.9-13 In vitro studies in which adherent layers from Dexter bone marrow cultures were exposed to lithium prior to ablation of active hematopoiesis by irradiation demonstrated that these “stromal” layers had an enhanced capacity to support in vitro hematopoiesis and suggested that lithium stimulates Dexter culture hematopoiesis indirectly by an effect on a radiosensitive adherent cell.12 Studies using the in vivo diffusion chamber system have also indicated that lithium acts indirectly, inducing a humoral factor that stimulates myelopoiesis.13 In 1980 Broxmeyer et al14 demonstrated that lithium is capable of abrogating lactoferrin-mediated inhibition of monocyte granulocyte–macrophage colony stimulating activity (GM-CSA) production. These results together with other findings15-16 have fostered the proposal that lithium acts via a macrophage or T cell mechanism to alter GM-CSA production. Chatelain et al17 have suggested that lithium’s action is on an accessory cell population requiring T lymphocyte function to produce stimulatory activities that then cause increased granulopoiesis and megakaryocytopoiesis. These studies indicate that lithium acts indirectly via a secondary cell population.

We have utilized liquid Dexter cultures18 of murine marrow to elucidate the mode of action of lithium. Previous studies have established that when lithium is added to this system the number of pluripotent stem cells, granulocyte-macrophage progenitors, macrophages, granulocytes, and megakaryocytes are increased.19 Lithium has also been shown to stimulate other hematopoietic precursors in Dexter culture: the colony-forming unit diffusion chamber (CFU-D)19 and the high proliferative potential colony-forming cell (HPP-CFC).21 In the present studies we have used the Dexter culture system to investigate the action of lithium on megakaryocytopoiesis. Furthermore, by using antibodies directed against various well-characterized growth factors, we have identified the cytokine responsible for the stimulatory activities of lithium in the Dexter system.

MATERIALS AND METHODS

Female C57Bl/6J, BDF, (Jackson Laboratories, Bar Harbor, ME) and ICR (Dominion Laboratories, Dublin, VA) mice 8 to 14 weeks of age were used as marrow donors in these experiments. Liquid bone marrow cultures. Liquid cultures were established using a modification of the method of Dexter et al,24 as previously published.25 Corning tissue culture flasks (25 cm²) were inoculated with bone marrow cells in 10 mL Fischer’s media with penicillin-streptomycin-fungizone, 10⁻⁵ mol/L hydrocortisone and 20% horse serum. Cultures were established by either flushing the marrow contents of a single tibia and femur directly into each flask or seeding each flask with 40 x 10⁶ cells from a pooled single cell suspension. The cultures were incubated at 33°C and 5% CO₂ and refed weekly by removing half the volume from each flask and replacing it with fresh medium. The nonadherent (or supernatant) cells were thus collected each week and were pooled for total cell counts and differential counts. The adherent (or stromal) cells were collected at different weekly time points by trypsinization using a 0.5% trypsin-EDTA solution (GIBCO, Grand Island, NY).26 The reaction was stopped by adding cold Hanks’ balanced salt solution.
with 5% fetal calf serum (FCS). A single-cell suspension was made and
total counts and differentials carried out.

In experiments in which megakaryocytic progenitor cells were
assayed, cultures were established with either 0 or 4 mEq/L lithium
chloride (Sigma Chemical Co, St Louis). The same amount of
lithium was added weekly in the medium used to refeed the cultures.
The supernatant and stromal cells collected weekly were pooled from
culture and refed with 10 mL fresh media, with or without 4 mEq/L
lithium was added weekly in the medium used to refeed the cultures.

Some cytospin slides were stained with acetylcholinesterase, a
specific marker for murine megakaryocytes, and the number of
positively stained cells were also determined.

Clonal agar assay. A modification of the soft agar culture
technique of Williams and Jackson was used to assay bone marrow
progenitor cells, as described previously. A supplemented McCoy's
5A media with 1 x 10^-4 mol/L 2-mercaptoethanol and 15% FCS was
employed for cloning the marrow cells in single layer cultures in
35-mm² petri dishes. When megakaryocyte precursors (CFU-meg)
were assayed, the stimulus used was either pokeweed mitogen
spleen-conditioned media (PWM-SCM) or interleukin 3 (IL 3; courtesy
of Dr James Ihle, Frederick, MD). The culture dishes were incubated
for seven days at 37°C and 5% CO₂, fixed with 10%
formalin, and whole agar slide preparations were made. The slides
were then stained with acetylcholinesterase and counterstained with
hematoxylin. Megakaryocyte colonies were scored as aggregates of
three or more acetylcholinesterase-positive cells.

Assays for stimulatory factors induced by lithium. Liquid
Dexter cultures were depleted of nonadherent cells at 3 weeks of
culture and refed with 10 mL fresh media, with or without 4 mEq/L
lithium chloride. The stromal layers were then exposed to 0 or 1,100
rad using a Gamma cell 40 machine delivering 120 to 128 rad/min.
Conditioned media (cm) was collected at various time points after
the addition of lithium and irradiation: 1, 2, 4, and 7 days, and after
1 and 2 days following the seven-day refueling of the cultures. The
cm was assayed for factors stimulating bone marrow progenitor cells
using the clonal agar culture system described above. Concentrated
(Amicon YM10 membranes, Amicon Corp, Lexington, MA) cm was
mixed directly in the McCoy's agar layer, while unconcentrated
cm was added in volumes up to 1 mL on top of the pregelled agar
plates. The culture dishes were incubated at 37°C, 5% for seven
days.

Alternatively, bone marrow stimulatory activity was assayed
using an in situ agar overlay system. In this technique the adherent
cells in the Dexter flasks are directly overlaid with two separate
layers of McCoy's nutrient media mixed with agar, with fresh
marrow target cells included in the top layer. The cultures were
incubated for seven days and scored for total colony growth.

The cm was also assayed for stimulatory activity using the
FDC-P1 or 32D cell lines (courtesy of Dr James Ihle, Frederick,
MD). Tritiated thymidine incorporation (³H-TdR) into the cells was
measured following a 24-hour exposure to the cm. The cell lines
were also used as the target cell population in the clonal agar assay
described above, and colonies of greater than 20 cells were scored
after seven days of incubation at 37°C and 5% CO₂.

Lastly, the conditioned medium was assayed for colony-stimulating
factor-1 (CSF-1) by radioimmunoassay (RIA; courtesy of Drs
Richard Shadduck and Abdul Waheed, Pittsburgh).

Antibody blocking of cm. The ability of polyclonal antibodies
against recombinant murine GM-CSF or IL 3 to inhibit FDC-P1 or
bone marrow colony proliferation induced by the Dexter culture cm
was assayed by adding the antibodies directly to the agar cultures in
Terasaki plates (FDC-P1) or in 96-well Costar plates (bone mar-
row). In the experiments utilizing Terasaki plates, 4 μL of McCoy's
media and agar mixture containing 2,000 FD cells were added into
each well, allowed to gel, and followed by 4 μL of 1x or 5x cm and 4
μL of antibody at a final dilution of 1:15 to 1:60. Normal rabbit
serum at the same dilution was added as controls. Colonies of greater
than 20 cells were counted after four and seven days of incubation at
37°C and 5% CO₂. For the 96-well plates, 60 μL of McCoy's media
and agar mixture containing 12,000 bone marrow cells was added
into each well, and followed by the same volume of 5x cm and
antibody at a final dilution of 1:120. Antibodies against purified
CSF-1 were also added into the bone marrow cultures at a final
dilution of 1:48. The various antibodies were titrated against their
purified growth factors for their capacity to inhibit growth factor-
stimulated cell growth. The dilutions described above completely
neutralized the growth stimulatory effects of the appropriate puri-
fied growth factors, that is, purified IL 3, purified CSF-1, or purified
recombinant Escherichia coli-derived murine GM-CSF.

The antibodies against recombinant GM-CSF or IL 3 were
obtained by Biogen, as previously reported. Purified CSF-1 and the antibody to purified CSF-1 were kindly
provided by Dr Richard Shadduck (Pittsburgh).

RESULTS

Effect of lithium on Dexter culture cell recovery. The
addition of 4 mEq/L lithium chloride to the long-term
Dexter cultures enhanced cell number and total megakaryo-
cytes over 6 weeks of culture (Fig 1). Megakaryocytes were
determined by differential counts of Wright's-Giemsa-
stained–cytospin preparations and were confirmed by counts
performed on acetylcholinesterase-stained slides.

The adherent cell populations were also increased in the
lithium flasks (Table 1). Total stromal cell and megakaryo-
cyte recovery were higher in the lithium groups at weeks 3
and 6.

Effect of lithium on megakaryocyte precursors (CFU-
meg). Dexter cultures containing 4 mEq/L LiCl had more
megakaryocyte progenitor cells (CFU-meg >3 cells) than
culture controls (Fig 2). Similar results were seen when the number of CFU-meg of over 20 cells was analyzed (data not shown).

Stromal CFU-meg (>3 cells) were also increased in the
lithium flasks, as shown in Table 1. Comparable results were
seen when colonies of greater than 20 cells were counted.

The supernatant and stromal cells were washed twice
before plating for CFU-meg to remove residual lithium. We
also carried out several experiments adding LiCl directly into
the soft agar system to rule out any possible effect of lithium
carry-over. LiCl was added at final concentrations of 0.0001
to 4 mEq/L into agar cultures containing plateau levels of
PWM-SCM, and no augmentation of megakaryocyte, gran-
ulocyte, macrophage, or mixed lineage colony formation was
seen (data not shown). A modest inhibition of CFU-meg was
seen at the higher concentrations of LiCl, 0.8 to 4 mEq/L.

The induction of hematopoietic growth factors by lihi-
um. Five separate experiments were carried out to deter-
mine whether lithium was inducing growth factor production
from the Dexter cultures. The addition of lithium was delayed until week 3 of culture, when a well-established
adherent layer had formed. Lithium was then added into flasks that received 0 or 1,100 rad irradiation. When the adherent layers in the culture flasks were overlaid with bone marrow target cells, the number of colonies stimulated by the lithium flasks was significantly higher than that seen in control flasks for both the unirradiated and irradiated groups (data not shown).

Conditioned medium harvested from the flasks at several time points following irradiation and the addition of LiCl (one, two, or seven days) had increased stimulatory activity for bone marrow colony formation. Figure 3 shows the total number of colonies, or colony-forming units in culture (CFU-C), stimulated by cm harvested 24 hours after the addition of 0 or 4 mEq/L LiCl to the flasks. Panel A shows the increase in bone marrow stimulating activity in the lithium cm over control for the unirradiated groups, and Panel B shows a similar effect of lithium in the irradiated groups. The breakdown of colony types from this same experiment is shown in Figs 4 and 5. The lithium cm stimulated higher numbers of granulocyte, macrophage, mixed granulocyte–macrophage, and megakaryocyte colonies than the control cm in both the unirradiated (Fig 5) and irradiated (Fig 4) groups. CSF-1 levels were not increased by lithium treatment of either normal or irradiated stroma (Table 2). This confirms other experiments showing no effect on CSF-1 levels when 4 mEq/L LiCl was added to normal Dexter cultures out to 6 weeks of growth (data not shown).

**Lithium induction of factors stimulating FDC-P1 cells.** To examine if the cm from the Dexter cultures contains GM-CSF or IL 3, the cm was assayed for activities that stimulate the FDC-P1 cell line. CM harvested from the flasks one and seven days after the addition of 4 mEq/L LiCl had increased FDC-P1 stimulatory activity whether control or irradiated stroma were evaluated and, as we have previously shown, irradiation per se increased FDC-P1 stimulatory activity. The stimulation of FDC-P1 3H-TdR incorporation by conditioned medium from lithium exposed stromal populations is shown in Fig 6. Similar results were seen in three

#### Table 1. Stromal Cell Recovery From Long-term Dexter Cultures

<table>
<thead>
<tr>
<th>Week</th>
<th>Group</th>
<th>Total Cells/Flask $\times 10^6$</th>
<th>Megakaryocytes/Flask $\times 10^3$</th>
<th>CFU-meg/Flask $\times 10^3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>Control</td>
<td>7.0 ± 1</td>
<td>7.65 ± 3.9</td>
<td>3.8 ± 2.8</td>
</tr>
<tr>
<td></td>
<td>Lithium</td>
<td>9.84 ± 0.8</td>
<td>39.80 ± 7.1</td>
<td>9.4 ± 4.3</td>
</tr>
<tr>
<td>6</td>
<td>Control</td>
<td>7.1 ± 2.8</td>
<td>12.10 ± 0.5</td>
<td>1.8 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Lithium</td>
<td>8.0 ± 1.5</td>
<td>16.60 ± 6.2</td>
<td>2.2 ± 0.4</td>
</tr>
</tbody>
</table>

Week 3 total cell counts are pooled from four separate experiments, and week 3 CFU-meg counts are pooled from three experiments. Week 3 megakaryocyte counts and all week 6 counts are from two separate experiments. Data are expressed as mean ± SEM.
LITHIUM AND GROWTH FACTORS

Fig 3. Assay for marrow colony-stimulating activity using conditioned medium from week 3 Dexter cultures after a 24-hour exposure to 0 (control) or 4 mEq/L lithium chloride (LiCl). (A), Dose-response curve of conditioned medium from nonirradiated (OR) cultures; (B), same dose range using conditioned medium from irradiated (1,100 rad) cultures. Colony-stimulating activity levels are expressed as mean ± SEM of total colony counts per 1 x 10⁶ bone marrow target cells. These data are from one representative experiment and were confirmed using conditioned medium from four separate Dexter culture experiments.

experiments when FDC-PI cells were stimulated by the cm to form colonies in agar.

In experiments in which a full-dose range of LiCl was added directly into the ³H-TdR assay, there was some stimulation of the FDC-PI cells by the LiCl alone at the higher dose levels (0.5 to 4 mEq/L). However, when these levels of LiCl were added to 1x cm from the control or 1,100-rad groups, there was essentially no augmentation of FDC-PI stimulation over that seen by either cm alone (data not shown). This demonstrated that the stimulating effect of the lithium cm on the FDC-PI cell line was not due to the LiCl acting directly on the cells.

Antibody blocking experiments. The FDC-PI cells are known to respond to both IL 3 and GM-CSF.28,30 Accordingly, experiments were performed assessing the effects of antibodies against each growth factor on cm-induced cell proliferation. Two clonal agar assays of the FDC-PI cells in Terasaki plates showed 98% to 100% inhibition of colony formation by the antibody to rGM-CSF in all four groups of cm assayed (Fig 7). FDC-PI colony growth was inhibited 0% to 9% by the antibody to rIL 3 using the lithium cm from both normal and irradiated flasks (data not shown). Previous

Fig 4. Morphology of colony types stimulated by irradiated culture-conditioned medium from the same experiment presented in Fig 3B. (A), Granulocyte; (B), macrophage; (C), mixed granulocyte/macrophage; (D), megakaryocyte. (●), 1,100 R control; (○), 1,100 R LiCl.

Fig 5. Morphology of colony types stimulated by nonirradiated culture-conditioned medium from the same experiment presented in Fig 3A. (A), Granulocyte; (B), macrophage; (C), mixed granulocyte/macrophage; (D), megakaryocyte. (●), OR control; (○), OR LiCl.
data had shown that the activity from the cm from normal or irradiated flasks (1,100 rad) was not blocked by antibodies against purified IL 3. The inhibition of stromal cm stimulation of FDC-Pl cells by antibody to rGM-CSF was confirmed in experiments utilizing "H-TdR incorporation into FDC-Pl cells as a measure of proliferation. Further evidence that the activity stimulating the FDC-Pl cells was GM-CSF was shown by the lack of stimulation by the cm of the cell line 32D, a cell line that responds to IL 3 but not to GM-CSF.

When the antibody to rGM-CSF was added to clonal agar assays with murine bone marrow cells as the target population, partial inhibition of colony growth was seen that was most prominent with the 1,100 rad Li group (Fig 8). Antibodies to CSF-i added in this same experiment also showed partial inhibition of the marrow stimulating activity, while wells containing the combination of the two antibodies showed 71% to 99% inhibition of colony growth (Fig 8).

In all of the antibody blocking experiments, controls were always included that showed 100% blockage of plateau levels of purified CSF-i by anti-CSF-i, purified recombinant GM-CSF by anti-GM-CSF, and purified IL 3 by anti-IL 3.
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There was no background stimulation of the bone marrow or FDC-Pl cells by the antibodies or normal rabbit serum alone. The antibody to rGM-CSF was selective in blocking rGM-CSF alone and not CSF-1 or IL 3. The antibody to CSF-1, however, did show some cross-reactivity by blocking rGM-CSF by approximately 30%.

DISCUSSION

We have previously reported stimulation of in vitro Dexter culture myelopoiesis by lithium chloride. The levels of a number of differentiated progenitor cells, including CFU-S, GM-CFU-C, CFU-D, and HPP-CFC, were increased in response to LiCl. Further data indicated that these stimulatory effects may be indirectly mediated by a radioreistant marrow adherent cell and that stromal cells were sources of multilineage CSAs. The present studies (Table I) show that lithium also induces increases in CFU-meg, which confirms and extends our previous observations on lithium stimulation of megakaryocyte production. Investigations by several researchers have demonstrated both in vitro and in vivo effects of lithium on a variety of hematopoietic lineages, and a number of these studies have indicated that these effects might be mediated by influencing the production of molecules that stimulate or modulate hematopoietic stem cell differentiation.

Our present data (Figs 7 and 8) provide evidence that lithium stimulates in vitro Dexter culture myelopoiesis by inducing secretion and/or production of GM-CSF. GM-CSF partially purified from concanavalin A (Con A)-stimulated spleen cm or recombinant GM-CSF can stimulate megakaryocyte, granulocyte, and macrophage differentiation. These are the three major phenotypes found in murine Dexter culture. GM-CSF also exhibits effects at the CFU-S levels, and several groups have now presented evidence that GM-CSF may stimulate early erythroid stem cells. Thus, elevations of GM-CSF could explain most of the previous reports of lithium's action in vitro and in vivo on different hematopoietic lineages.

IL 3, another growth factor having a wide range of hematopoietic bioactivity, was a prime candidate as a regulator for Dexter culture hematopoiesis. However, the present data showing no effect of anti-IL 3 antibodies on stromal-derived growth factors and no effect of stromal cm on the IL 3 responsive 32D cell line strongly indicate that IL 3 is not a regulator in this culture system. As to CSF-1, although it is produced in Dexter cultures, we have not found elevations of this factor after irradiation, or lithium exposure, manipulations that all appear to increase GM-CSF production in this system. This may in part be because of binding and utilization of CSF-1 by adherent macrophages, the major cellular component of the stromal cells remaining after exposure to 1,100 rad. CSF-1, however, does not stimulate megakaryocytes or FDC-Pl cells and has a relatively small effect on granulocyte production. Our experiments showing virtually complete inhibition of cm-induced marrow cell proliferation by a combination of antibodies to CSF-1 and GM-CSF suggest that these two growth factors are the critical hematopoietic differentiating hormones controlling Dexter culture-differentiated cell production, but the stimulation of hematopoiesis seen in response to lithium appears due to a selective induction of GM-CSF secretion.

Lithium chloride may also induce other growth factors acting on earlier stem cells. Elevations of GM-CSF would not seem to account for the Li-induced stimulation of HPP-CFC. This latter cell requires CSF-1 and one of several "synergistic" activities, including IL 3 and Hematopoietin-1. We have isolated a cell line from Dexter-type cultures that produces CSF-1, GM-CSF, and a separate non-IL 3 synergistic activity that does not have intrinsic colony-stimulating activity but that synergizes with GM-CSF, CSF-1, and IL 3. Whether a similar factor may be important in normal Dexter culture hematopoiesis and whether lithium may also induce production of such a factor remains open questions. Preliminary studies, however, suggest the possibility that a CSF-1-synergistic activity that is not IL 3 might also be induced by lithium in Dexter cultures.

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