Lithium Stimulation of Murine Hematopoiesis in Liquid Culture:
An Effect Mediated by Marrow Stromal Cells

By Peter J. Quesenberry, Michael A. Coppola, Richard J. Gualtieri, Philip M. Wade, Zengxuan Song, Michael A. Doukas, Charlotte E. Shideler, Donald G. Baker, and Elizabeth H. McGrath

Lithium has previously been observed to stimulate in vitro Dexter culture hemopoiesis with increases in granulocytes, megakaryocytes, and pluripotent stem cells (CFU-S). In the present study, a two-phase murine Dexter culture system was established to study the mechanism of lithium-mediated stem cell stimulation. Different lots of horse sera or fetal calf sera were found to have markedly different effects on Dexter culture growth; given the appropriate sera supplementation, supernatant cells from Dexter cultures established from C57BL/6J mice 3 wk previously were free of stromal-forming capacity, but had stem cells and could grow on 900–950 R irradiated stroma. Conversely, in vitro irradiation (900–950 R) of 3-wk cultures resulted in a stem-cell-free adherent monolayer that could support growth for up to 9 wk in culture. The stroma from Dexter cultures preexposed to lithium chloride (1.0 mmole/liter) for 3 wk, irradiated (900 R), and then refed with 3-wk Dexter supernatant cells has an enhanced capacity to support cell production, CFU-S, and probably granulocyte-macrophage colony-forming cell (GM-CFU-C) production, as compared to stroma not preexposed to lithium. Lithium carryover was ruled out in these experiments. These data indicate that lithium stimulates CFU-S and in vitro granulopoiesis by an indirect effect on a radioresistant adherent stromal cell.

ADMINISTRATION of the monovalent cation, lithium, stimulates granulopoiesis, attenuates chemotherapy-induced myelosuppression in humans, and probably stimulates platelet production. Although it augments in vitro granulocyte-macrophage colony-stimulating activity (GM-CSA) production in both humans and mice, anecdotal reports of remissions in aplastic anemia coinciding with lithium therapy and observations that lithium may enhance both neutrophil and erythrocyte production in grey collie dogs with cyclical neutropenia suggested that lithium may be acting at a multipotent stem cell level rather than simply on granulocyte production. Our own studies, utilizing Dexter long-term liquid cultures of murine marrow, established that lithium acts at the pluripotent stem cell (CFU-S) level, with additional stimulatory effects on granulocyte-macrophage stem cells (GM-CFU-C) and granulocyte, megakaryocyte, and GM-CSA production. In addition, there was a late inhibition by lithium of all in vitro myelopoiesis in this culture system. Further studies in in vivo murine models have shown that lithium expands the pluripotent stem cell compartment, and other data have suggested that lithium may act via a macrophage or T cell mechanism to alter GM-CSA production, while Broxmeyer and colleagues have shown that it is capable of abrogating lactoferrin-mediated inhibition of monocyte CSA production. Recently, Chatelain et al. suggested that lithium enhancement of early megakaryocytopenia and granulopoiesis may be due to local production of colony-stimulating activity by accessory cell populations requiring the integrity of T-lymphocyte function. In order to further elucidate the mode of action of lithium on Dexter culture hemopoiesis, we have established a two-phase system in which a nonhemopoietic stromal phase and a non-
with 10 ml of culture media. Alternatively, marrow cell suspensions from C57BL/6J mice were first prepared and standardized to equivalent cell concentrations in 10 ml of medium before inoculation. All cultures were incubated at 33°C and 5% carbon dioxide. At weekly intervals, half the volume of each flask was removed, and a similar amount of fresh medium was added. Pooled suspensions from the flasks were then counted, stem cells assayed, and slides made for subsequent staining with Wright-Giemsa.

Stromal cell analysis was carried out visualizing the extent and fat content of adherent cells under a dissecting microscope. In order to quantitate the stroma, it was removed and analyzed as follows: all supernatant cells (10 ml) were removed, the stroma washed twice with Hanks’ balanced salt solution, and then just enough (1-1.5 ml) Trypsin-Versene 0.4% (Microbiological Associates, Bethesda, MD) to cover the stroma added to each flask and incubated at room temperature for approximately 3 min with intermittent agitation (duration of incubation dependent on both gross and microscopic dissolution of the stroma). Hanks’ balanced salt solution with fetal bovine serum was added to terminate the reaction, and the entire eluate aspirated from the flask. Total counts and differentials were then carried out.

Lithium chloride (MW 42.39, Fisher Scientific, Pittsburgh, PA), after dissolution in aliquots of Dexter culture medium, was added to flasks when cultures were first established. Final concentrations of lithium in the supernatant ranged from 1 x 10^{-4} to 8 mmole/liter. In general, calculated lithium concentrations correlated closely with measurement by atomic absorption spectroscopy, although, in a few instances, discrepancies were noted. Five to ten culture flasks were established for each lithium concentration, and a similar number of flasks was inoculated with an identical aliquot of medium without lithium and served as concomitant controls. Flasks were replenished at weekly intervals with Dexter medium and aliquots of lithium to maintain the concentrations of lithium in the supernatant, unless the experimental design called for refedding with lithium-free media (see below). Cell concentrations in pooled supernatants were calculated after hemocytometer counts; differentials were determined from 154 to 809 cells per count after Wright-Giemsa staining of cytocentrifuge slide preparations. Cells were washed three times in Hanks’ balanced salt solution prior to assay for CFU-C and CFU-S.

**Irradiated, Lithium Preexposed, Stroma Experiments**

Experiments established that, with our lot of horse serum (Flow lot 29211086), supernatants from Dexter cultures at 3 wk or longer of culture and at a cell inoculum ranging from 0.5 to 6.5 x 10^{6}/flask could not form stroma or grow in blank flasks but could grow quite well in the presence of 900–950 R exposed stroma.

Similarly, it was found that 900–950 R stroma did not produce hematopoietic cells, but supported in vitro hematopoiesis quite well (see Results section). This “biphasic system” was utilized in experiments designed to test the effect of lithium on the hematopoietic support capacity of radiosensitive stromal cells. Dexter cultures were established with 0 or 1 mM lithium chloride, demidepopulated for 3 wk, and at 3 wk, all supernatant cells removed. The stroma were then washed 6–8 times with 10 ml per wash of Dexter culture media without lithium. These washed stroma were exposed to 900–950 R x-irradiation and then refed with control supernatant cells (0.22–1.65 x 10^{6}/flask) from the same experiment or from other experiments at 3 wk or more of culture. All cultures, lithium preexposed and control, were then refed weekly with Dexter media without lithium, and cell counts and CFU-C and CFU-S assays were carried out as described above.

**Lithium Levels**

The measurements of lithium concentrations present in the culture media were done by atomic absorption spectrophotometry. A lithium hollow cathode lamp and a Model 751 Instrumentation Laboratory atomic absorption/atomic emission spectrophotometer were used (Instrumentation Laboratory, Inc., Wilmington, MA). Lithium was measured using the 670.8-nm resonance line with a 1.0-nm bandpass. A slightly oxygen-rich, air-acetylene flame was used with a 10-cm single slot burner. Nominal aspiration rate was approximately 7.0 ml/min.

Working standards for the lithium analyses were prepared from a stock 144.1 mmole/liter lithium standard (Fisher Scientific). Dilutions of the stock standard were made with deionized H_{2}O. Standards ranged in lithium concentration from 0 to 2 mmole/liter.

The standards and samples were diluted 1:16 with 5% trichloroacetic acid, mixed thoroughly, allowed to stand for 5 min, and spun for 10 min at approximately 800 g in a table-top centrifuge to sediment the protein. The protein-free supernatants were aspirated into the flame of the atomic absorption spectrophotometer setup and optimized for lithium measurements. The absorbances of the samples were translated to lithium concentrations using the absorbance curve prepared from the standards.

**Statistics**

Statistical analyses were performed by means of an unpaired t-test, or a two-sample test.

**RESULTS**

**General Aspects of System**

**Serum Effects**

In initial experiments evaluating the effect of lithium chloride on Dexter hemopoiesis and using a different lot of horse sera than was used for the experiments previously reported, we found different kinetics of growth stimulation by lithium. Previously, we had observed that 1 mmole/liter lithium chloride gave an early stimulation (approximately 3 wk) and a late inhibition of cell recovery in Dexter culture; now, at the same lithium level, we saw a more pronounced and prolonged stimulation without the late inhibition (Fig. 1). At 4 meq/liter, however, the same early stimulation and late inhibition was seen. These differences appear to be based on the marked differences in growth seen with different lots of horse serum. The growth of marrow from C57BL/6J mice in Dexter cultures showed marked differences, dependent on the lot of horse sera (6 lots tested) or fetal calf sera (4 lots tested) utilized. Stromal grading correlated poorly with supernatant cell recovery. Secondary cultures were established with 3 x 10^{6} or 10 x 10^{6} cells from 3-wk cultures, and the effect of these sera on secondary culture growth was also evaluated. In general, there was little sustained growth after 1 wk of secondary culture and little stromal formation. However, one lot of horse serum supported secondary growth quite well (HS 222100), although the stroma was atypical, consisting of many individual single cells. The experiments...
LITHIUM STIMULATION OF HEMATOPOIESIS

Fig. 1. Effect of lithium chloride (1 meq/liter) on total cell recovery in Dexter culture of C67BL/6J murine marrow expressed as the mean percent of control ± 1 standard error of the mean. Numbers in parentheses represent number of separate experiments evaluated, with 5 flasks/experimental point in each experiment.

outlined below were carried out with HS 29211086 from Flow Labs.

Lithium Effects on Stroma

Both 1 and 4 mmole/liter lithium chloride increase the total number of adherent cells in Dexter culture. At 0 and 4 mmole/liter, the mean number of adherent cells per culture at week 3 were 4.07 ± 0.62 × 10⁶ and 7.125 ± 2.01 × 10⁶, respectively (4 experiments), and at week 4, 6.37 ± 1.05 × 10⁶ and 15.413 ± 0.7 × 10⁶, respectively (2 experiments).

Biphasic System

Hemopoietic Stem Cell Populations Without Stroma-Forming Capacity

A number of approaches were utilized to attempt to selectively remove stromal-forming cells from hemopoietic stem cells. These included repeated short-term (hours to days) adherence separations, density separations on Hypaque-Ficoll, or cryopreservation; these were unsuccessful. However, long-term culture in supplemented Dexter media with horse serum (lot 2921 1086), for 3 wk or longer, resulted in a supernatant population that had stem cells and could grow on irradiated preformed stroma, but which could not form stroma or grow in blank flasks (Table 1). These cells, at up to 10 × 10⁶ cells/flask, were unable to establish stroma or grow in blank flasks. Similarly, supernatant cells at 4 or 5 wk (or longer) could be used as a source of hemopoietic stem cells that could not grow in blank flasks or establish an adherent stromal layer.

Adherent Stromal Populations Without Hemopoietic Stem Cells

The techniques described above were also assessed for their capacity to establish an isolated stroma phase; none proved successful. Multiple attempts at "washing" out stem cells from adherent stroma of Dexter culture at 3 wk of culture or longer were unsuccessful, and these experiments usually terminated in infection without any appreciable exhaustion of hemopoietic proliferative capacity. Exposure of the stroma to 900–950 R, however, effectively eliminated hemopoiesis but preserved the hemopoietic supporting capacity of the stroma. The effect of graded doses of x-irradiation at 3 wk of culture on subsequent cell production in Dexter culture is shown in Fig. 2. At 3 or 4 wk of culture, 900–950 R reproducibly reduces cell numbers at 1 wk postirradiation to 1.1% ± 0.03% of the nonirradiated cultures (total of 10 experiments); the majority of these residual cells are macrophages. The capacity of 950 R stroma to support the growth of 3-wk nonadherent cells as compared to growth in blank flasks is illustrated in Fig. 3. These data indicate that irradiation...
Fig. 2. In vitro radiation. Dexter cultures of C57BL/6J demidepopulated for 3 wk, stroma exposed to 0, 250, 750, or 950 R, and flasks refed with blank cell-free media.

The radiation of adherent cells at 3 wk of culture results in a population of cells that can support Dexter culture hemopoiesis but which is effectively sterilized of hemopoietic stem cells. Other studies have shown that recognizable hemopoietic stem cells are selectively depleted by irradiation, leaving basically two cell types. A morphological and functional characterization of these cells is presented in these studies. Thus, the “stromal” phase of the biphasic system consists of irradiated adherent cells from 3-wk Dexter cultures. Similarly, supernatant cells from 3-wk Dexter cultures provide the “hemopoietic” phase of the biphasic system.

Fig. 3. Week 3 supernatant cells grown on 900 R stroma or blank flasks. Supernatant cells from Dexter cultures established 3 wk previously with C57BL/6J marrow were used to attempt to reestablish Dexter culture hematopoiesis in blank flasks or on 3-wk irradiated (900 R) stroma. “Weeks” refers to the number of weeks since original cultures were established. 6.5 x 10^6 or 0.65 x 10^6 cells were implanted into blank flasks and 0.65 x 10^6 cells into flasks with 900 R exposed stroma. 900 R alone without cells produced negligible numbers of cells at 1 wk postirradiation. Data are expressed as number of cells per flask x 10^6.

Fig. 4. Table 2. Absolute Values for Total Cell and Stem Cell Recovery From Dexter Cultures Before and After Stromal Irradiation and Refeeding in Control Flasks

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*Original cultures. Not exposed to lithium.
†Cultures of control supernatant cells from 3-wk Dexter cultures at from 0.22 x 10^6 to 1.65 x 10^6 cells per flask grown on irradiated (900R) control stroma. In one experiment, in addition to nonadherent 3-wk Dexter supernatant cells, stroma underlayers were overlayed with 0.05 x 10^6 light density Hypaque-Ficoll-separated C57BL/6J marrow cells.

Effect of Lithium Preexposure on the Hemopoietic Supportive Function of Dexter Adherent Cells

We utilized the biphasic system described above to investigate the effect of preexposure to lithium of adherent cells on the subsequent capacity of those adherent cells to support in vitro liquid culture hemopoiesis. Dexter cultures were established from tibia and femur blowouts and exposed to 0 or 1 mM lithium chloride for 3 wk of culture. At 3 wk, all supernatant cells were removed, the stroma washed extensively, and exposed to 900 R x-irradiation. The control or lithium-preexposed washed irradiated stroma were

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then refeed with supernatant cells that had never been exposed to lithium from long-term Dexter cultures (3 wk or more) at levels ranging from 0.22 x 10^6 to 1.65 x 10^6 cells per flask. Table 2 presents the absolute total cell, CFU-S, and GM-CFU-C recovery per flask in the control groups in these experiments. The effect on total cell recovery in these refeeding experiments is presented in Fig. 4, showing a lithium-induced stimulation of total cell recovery from 1-4 wk postirradiation and refeeding (significant at p < 0.05 at 1 wk after irradiation). There was no significant shift in differentiation patterns, as assessed by granulocyte and macrophage counts, between the control and lithium-exposed stromal groups (Table 3). The effect on CFU-S and CFU-C recovery after irradiation and refeeding is presented in Fig. 5. There was an early stimulation of CFU-S (significant at p < 0.05 at 2 wk after irradiation [RT]), followed by a later increase in CFU-C numbers per flask in the lithium-pretreated groups as compared to control stromal groups.

Lithium Levels

In 64 of 65 determinations, lithium was undetectable in supernatants from control flasks, while in 1 experiment, a level of 6.2 x 10^-3 meq/liter was found. In flasks with a calculated lithium level of 1 meq/liter, the measured values were 1.111 ± 0.107 meq/liter (62 determinations on separate groups). At 1-8 wk after washing, irradiation, and refeeding of lithium-preexposed stroma layers, there was no detectable lithium in 19 of 23 samples. The 4 positive values were from one experiment and ranged from 1.2 x 10^-3 to 3.7 x 10^-3 meq/liter at 1-4 wk after irradiation and washing. Over the same time intervals, 22 of 23 control flasks had no detectable lithium, while one flask had a value of 2.9 x 10^-3 meq/liter (a value close to the limit of detection).

Lithium Dose Response

The effect of lithium chloride at levels from 8.0 to 1 x 10^-4 mM on Dexter culture growth was assessed,
experiments per time point. There were 3-5 flasks per experiment. The mean numbers of cells ±

4. 5, and 6 wk of culture were 3.7 ±

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DISCUSSION

The stimulatory effect of lithium on granulopoiesis

was an initial focal point of investigation,14 and presumptive mechanisms involving GM-CSA were proposed.89 A number of studies of in vitro liquid culture

or in in vivo systems now suggest that lithium acts on relatively primitive multipotent stem cells.13-15 The present observations indicate that lithium exerts an effect on radioresistant adherent cells that leads to stem cell stimulation. This effect appears operative at the CFU-S level, and, considering the kinetics of growth, the effects on CFU-C and differentiated cell production could be interpreted as secondary. These observations, of course, do not rule out the possibility of effects at multiple levels of cellular differentiation. Preliminary results, in fact, suggest that lithium preexposure of stroma results in augmented GM-CSA levels postirradiation.10 This could represent an epiphenomenon or simply be one of many factors produced by lithium-preexposed stroma. The latter possibility is supported by observations indicating that irradiated stroma produces factors stimulating not only GM-CFU-C but also megakaryocyte colonies, mixed granulocyte-megakaryocyte colonies, and blast colonies.29 Thus, lithium may induce production of stromal-derived factors acting at multiple, relatively primitive, stem cell levels. The present studies do not rule out concomitant direct effects on hemopoietic stem cells, but they do indicate that lithium can exert these effects indirectly by acting on adherent stromal cells. These studies also do not rule out the possibility that lithium may also exert effects on radiosensitive adherent cells.

Overall, however, these data suggest that lithium induces radioresistant stromal cells to produce factors active at the CFU-S level. This speculation is supported by observations that lithium stimulates several other putative primitive hemopoietic stem cells in Dexter culture: the colony-forming unit diffusion chamber (CFU-D)31 and the high proliferative potential colony-forming cell (HPP-CFC).32 An in vivo model also exists for the apparent indirect effect of lithium on hemopoietic stem cells.31 In this model, mice received a series of preinjections with lithium chloride for 7 days, and, 4 days after the last injection, were implanted with plasma clot diffusion chamber marrow cultures; when the chambers were harvested from the peritoneum 4 days later, a marked stimulation of colony formation (CFU-D) in the plasma clots was seen. However, if the lithium was administered during the period of diffusion chamber culture, no stimulation occurred. These observations suggest that these in vitro observations may have in vivo physiologic relevance.

Our observation of the relative radioresistance of the hemopoietic supportive capacity of adherent stromal cells in Dexter culture is at variance with reports of others.33,34 This may in part be due to the possibility that previous reports observed both stromal function and residual hemopoietic stem cell damage. We have found that the stromal hemopoietic supportive capacity is preserved after in vitro irradiation to levels of 900-950 R. Furthermore, marrow from mice exposed to 1,000 R in vivo irradiation immediately prior to establishing Dexter culture are capable of forming adherent cell colonies that will support hemopoiesis and CFU-S in liquid culture.28 We have observed, as have others,35 that basic characteristics of long-term Dexter marrow culture are markedly altered by the lot or type of serum utilized in the culture system. Unfortunately, until adequate serum-free systems are devised, this will remain an important limiting feature of this culture system, making comparisons between laboratories difficult.

<table>
<thead>
<tr>
<th>Group (meq LiCl/Liter)</th>
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<th>3</th>
<th>4</th>
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<th>6</th>
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<td>0.0001</td>
<td>85 ± 14 (7)</td>
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<tr>
<td>0.001</td>
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<td>88 ± 40 (3)</td>
<td>88 ± 27 (3)</td>
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<tr>
<td>0.01</td>
<td>97 ± 3 (2)</td>
<td>114 ± 46 (3)</td>
<td>113 ± 32 (2)</td>
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<td>4.0</td>
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<td>168 ± 19 (5)</td>
<td>280 ± 55 (5)</td>
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<tr>
<td>8.0</td>
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*Cell/flask expressed as a percent of control ± 1 standard error of the mean (SEM). Numbers in parentheses represent the number of separate experiments per time point. There were 3-5 flasks per experiment. The mean numbers of cells ± 1 SEM per flask × 10^6 in the control groups at 1, 2, 3, 4, 5, and 6 wk of culture were 3.7 ± 0.4, 1.3 ± 0.2, 1.8 ± 0.4, 5 ± 0.2, 6.9 ± 0.2, and 1.2 ± 0.1, respectively.
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