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ABILITY OF LITHIUM TO ACCELERATE THE RECOVERY OF GRANULOPOIESIS AFTER SUBACUTE RADIATION INJURY

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Abstract

Lithium stimulates granulopoietic recovery after mice are exposed to 2 Gy. By examining the hematopoietic inductive microenvironment (HIM) using the stromal colony assay, we demonstrate here that lithium, during the two weeks after irradiation, produced less stromal colony suppression than was observed from the irradiated controls. Recovery peaked by day 19 and returned to normal by day 28. This response was also observed in splenic derived stroma. Furthermore, stroma from lithium-irradiated animals supported the in vitro growth of granulocyte-macrophage colonies (CFU-GM) greater than observed from irradiated controls. These data suggest lithium accelerates granulopoietic recovery by first providing for a completely reconstituted and functional HIM.

Lithium has been used as the treatment of choice in manic-depressive illness for many years (17). A side-effect induced in these patients was the appearance of a leukocytosis. It is this property of lithium that has stimulated interest in its use to ameliorate the neutropenia present after routine chemotherapy (2, 11, 14).

Because radiation protocols can and often develop neutropenic conditions which can also reduce the survival of patients by increasing the chance of infection, we have recently examined the ability of lithium to enhance granulopoietic recovery after mice were administered 2 Gy whole body irradiation (10). These studies were designed to investigate the feasibility of lithium to enhance granulopoietic recovery after total whole body irradiation. Bone marrow and spleen cells were examined for their recovery patterns of CFU-S, CFU-GM, CFU-E, BFU-E and per cent ^{59}Fe incorporation along with the usual peripheral blood indices (packed red cell volume, WBC and differential). Lithium increased granulopoietic recovery as measured by significant increases in both bone marrow and spleen derived CFU-GM compared with those values obtained from irradiated controls. Significant elevations in the WBC, consisting of neutrophils were also observed. Bone marrow and splenic derived erythroid stem cells (CFU-E, BFU-E) and per cent ^{59}Fe incorporation measured from peripheral blood, femur and spleen were all slightly reduced but not to a significant degree to alter the packed red cell volume. The CFU-S populations from both irradiated groups (control and lithium treated) were depressed when compared with the normal non-irradiated controls and this degree of suppression was greater in the lithium-treated group.

To further explore the manner by which lithium influences granulopoietic recovery after radiation-induced injury, we investigated the effect of lithium on the recovery of the hematopoietic inductive microenvironment (HIM) as measured by the stromal colony assay (21). In these investigations, we studied the ability of the (HIM) to support granulopoietic stem cell colony (CFU-GM) formation by examining for granulocyte-macrophage colonies obtained from non-adherent bone marrow placed in agar overlaid over the stroma cell colonies. In addition,

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we examined for any distinct morphologic change in the cell type constituting a CFU-GM colony by the use of specific cytochemical stains.

Materials and Methods

The experiments were performed on male mice of the B6C3F1 strain (C3H♀×C_{57}B46♂) which were between 10 and 12 weeks of age at the start of each experiment. They were housed in plastic cages and fed a diet of Purina lab chow and water ad libitum.

Hematologic methods. Following killing by cervical dislocation, femurs and spleens were removed and bone marrow and spleen cells were harvested. Single cell suspensions were prepared in McCoy's 5A medium (Gibco, Grand Island, NY) after repeated passage through a 25 gauge needle. After centrifugation (400 g, 10 min, 4°C), the cells were resuspended in McCoy's medium for routine cell counting with a Coulter Counter, Model ZBI (Coulter Electronics, Hialeah, FL).

Irradiation and lithium. Two hundred mice were administered total body irradiation (2.0 Gy at a dose rate of 0.7 Gy/min) using a Siemans Stabilipan 250 roentgen unit. The dose rate was measured in air with a Victoreen condensor R-meter at a target object distance of 73 cm. The physical factors used were 250 kV, 15 mA and a 2.0 mm Al filter. Immediately after irradiation and on the next two days, 100 irradiated mice were administered 0.035 mg/animal/day of ultrapure lithium carbonate (Alfa Products, Danvers, MA) intraperitoneally as previously reported from this laboratory (7). All vials, needles and syringes used were pyrogen free. This lithium preparation was free of endotoxin as determined by the limulus lysate assay. The lithium dose has been shown by us to be optimal for hematopoietic stimulation of (CFU-S and CFU-GM) both in vivo and in vitro (8). One hundred mice served as irradiation controls and 50 mice served as non-irradiated, non-lithium treated controls. All experiments were performed in triplicate. Beginning 24 hours after the last lithium injection and over the next 30 days, mice were randomly killed from each group and their bone marrow and spleen cells assayed for their stromal cell content by the following technique.

Assay for stromal colonies. A modification of the procedure of WERTS et coll. (21) was used to culture stromal colonies. Briefly, 2×10^6 marrow or spleen cells were placed in 5 ml of McCoy's 5A media containing 100 U/ml penicillin and 50 μg/ml streptomycin supplemented with 20% fetal bovine serum. Cultures were incubated for 9 days at 10% CO_2 in air in a fully humidified incubator. After the incubation period, the cultures were removed, the supernatants decanted and the cultures were then stained with Wright's stain for morphologic examination. Colonies which consisted of 30 or more macrophages/fibroblasts were enumerated.

Measurement of the functional capacity of the HIM. Studies were performed in order to assess the functional capability of the regenerating stroma to support CFU-GM colony formation. After the culture supernatants from the stromal assay plates were discarded, an agar layer (0.5%) was placed over the stromal colonies from the two groups of irradiated mice. Then this was overlayed with another agar layer (0.3%) containing non-adherent derived normal bone marrow cells. No exogenous source of colony stimulating factor was added. The plates were then reincubated for an additional 7 days when the plates were removed and assayed for their CFU-GM content using a dissecting microscope. Any colony consisting of 50 or more cells was scored as a CFU-GM.

Morphologic identification of stromal induced CFU-GM as indicated by cytochemical staining. In order to determine the exact morphologic type of CFU-GM generated by the stromal colonies, each culture dish was prepared as follows: The agar dishes were overlayed with No. 1 Whatman filter paper and heat dried until the agar became fixed to the base of the culture dish. At this time the filter paper was gently removed and the fixed agar dishes were stained separately for the presence of non-specific and specific esterase activity. Duplicate culture dishes per experimental point were examined to allow one set to be stained for non-specific and specific esterase activity, respectively.

Procedure for the cytochemical demonstration of naphthol AS-D chloroacetate esterase and α-naphthyl acetate esterase activity. The technique use to detect for naphthol AS-D chloroacetate and α-naphthyl acetate esterase was a modification of the procedure originally described by YAM et coll. (23). After the agar dishes had been sufficiently dried, they were fixed for 30 s to 2 min with a citrate-acetone-methanol fixative. Then the dishes were washed thoroughly with deionized water and were allowed to air-dry for 20 min. For the detection of naphthol AS-D chloroacetate esterase, 50 ml of diluted buffer solution was added to Fast Cornith

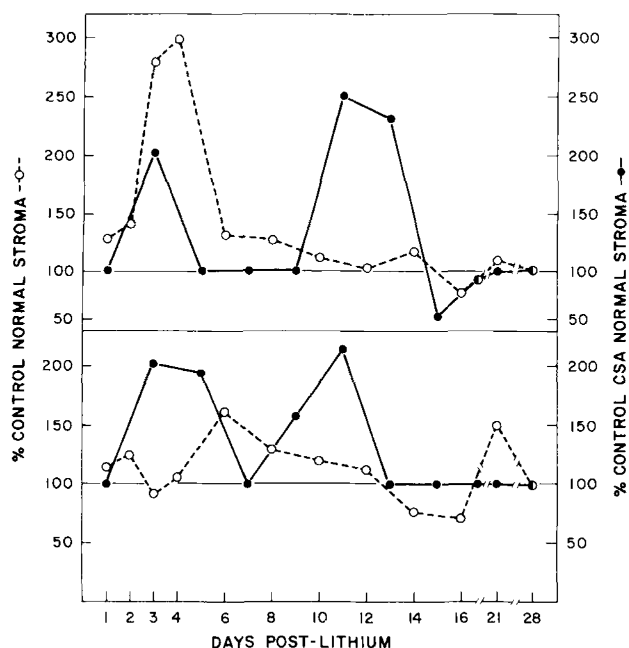

Fig. 1. The effect of lithium on the number of stromal colonies (○) derived from normal spleen (top) and normal bone marrow (bottom) and their ability to support normal non-adherent cell derived CFU-GM (●). Values expressed are the means taken from triplicate studies expressed as per cent of control.

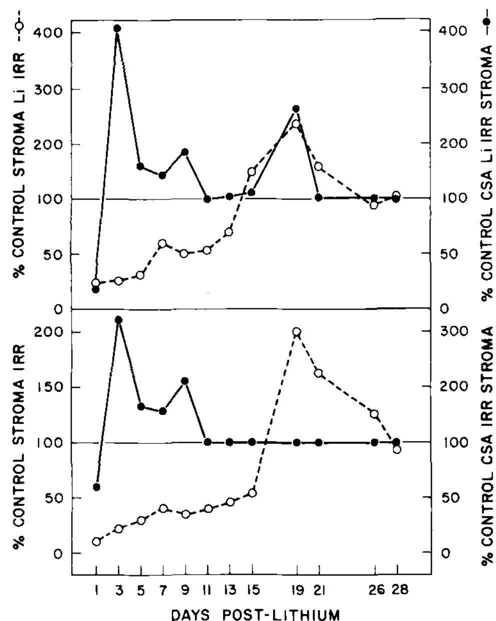

Fig. 2. The effect of lithium on the number of stromal colonies (○) derived from bone marrow taken from animals administered irradiation (2 Gy) and lithium (top) compared with the animals receiving irradiation only (bottom) and their ability to support normal non-adherent cell derived CFU-GM (●). Values expressed are the means taken from triplicate studies expressed as per cent of control.

Salt. When the contents were completely dissolved, 2 ml naphthol AS-D chloroacetate solution was added and was allowed to mix for 30 s. Sufficient staining material was then added to cover the area of the culture dish and then allowed to stand for 30 min at 37°C. For the detection of α-naphthyl acetate esterase, 50 ml of diluted buffer solution was added to Fast Blue RR salt. When the contents were completely dissolved, 2 ml of α-naphthyl acetate solution was added and was allowed to mix for 30 s. Sufficient amounts of staining material was then added to cover the area of the culture dish and was allowed to stand for 30 min at 37°C. After the 30 min was completed, the dishes were washed with deionized water and were counter-stained for 5 min with Mayer's hematoxylin. Cells positive for naphthol AS-D chloroacetate are specific for the granulocytic lineage and appear red, while cells positive for α-naphthyl acetate esterase which are primarily monocytes and/or macrophages appear black.

Statistical analysis. The significance of the difference between groups was evaluated by the one-tailed Student's t-test. A p-value of ≤ 0.05 was the level used as significant. All analyses were performed in triplicate.

Results

The ability of lithium to increase the number of stroma derived colony forming stem cells is given in Fig. 1. Lithium increased stromal colony formation both from bone marrow and spleen cells with the most dramatic increase observed from the spleen (300% of control on day 4, $p \leq 0.001$). The bone marrow response peaked on day 6 at 165 per cent of control ($p \leq 0.01$). In both groups, the number of stromal colonies gradually returned to normal levels by day 12 (spleen) and by day 13 (bone marrow). In addition, both bone marrow and spleen derived stromal colonies had the capacity to support the growth of granulocyte-macrophage colony forming stem cells (CFU-GM). However, what appears significant is the observation that the increase in stromal support of CFU-GM under the influence of lithium and/or irradiation did not necessarily correlate with the exact day when the number of stromal colonies was elevated above the normal control.

The ability of lithium to influence the recovery of the bone marrow derived stromal cell population after 2 Gy is given in Fig. 2. Although the number of bone marrow derived stromal colonies from both the irradiated and lithium-treated groups was re-

duced, the degree of suppression was less severe in the lithium irradiated group ($p \leq 0.05$). Lithium returned bone marrow derived stromal colonies to above normal control levels earlier than was observed from the irradiated group ($p \leq 0.005$). In addition, the stroma from both irradiated groups increased the support of CFU-GM colony formation above normal controls, however, the level of the lithium-stromal support was 100 per cent higher than compared with the irradiated group ($p \leq 0.01$). Also a second peak of response was observed from the lithium irradiated group that was not present in the irradiated controls (day 19, $p \leq 0.001$).

Lithium was also effective in stimulating the recovery of spleen derived stromal colonies as is depicted in Fig. 3. As was observed for the bone marrow, the degree of stromal colony suppression and time of recovery to normal control levels occurred earlier in the irradiated group receiving lithium when compared with the irradiated controls ($p \leq 0.001$). The peak effect induced by lithium was greater by approximately 80 per cent on day 15 ($p \leq 0.01$). The appearance of the splenic stromal support of CFU-GM colony formation was evident, as was observed from the bone marrow, however, from the spleen it occurred earlier. Peak increase was observed on day 3 when lithium increased stromal support of CFU-GM by approximately 100 per cent ($p \leq 0.01$).

The ability of lithium to influence the type of stromal colony is shown in the Table. Only in the presence of lithium did any colonies and/or cells associated with those colonies stain reactive for the specific granulocyte enzyme marker naphthol AS-D chloroacetate esterase. There was also an increase in the number of colonies staining with the enzyme marker for monocyte/macrophages non-specific esterase, α-naphthyl. This observation demonstrates the effectiveness of lithium to increase the number of cells and/or colonies associated with the formation of the hematopoietic stroma but also indicates that the ability of lithium derived stroma to support the growth of non-adherent cell derived CFU-GM colony formation may in part be due to the increased numbers of monocytes and/or macrophages, cells known to elaborate GM-CSF. Further studies are required to elucidate this effect of lithium.

Discussion

The normal steady-state regulation of hematopoiesis involves the interaction between active hemato-

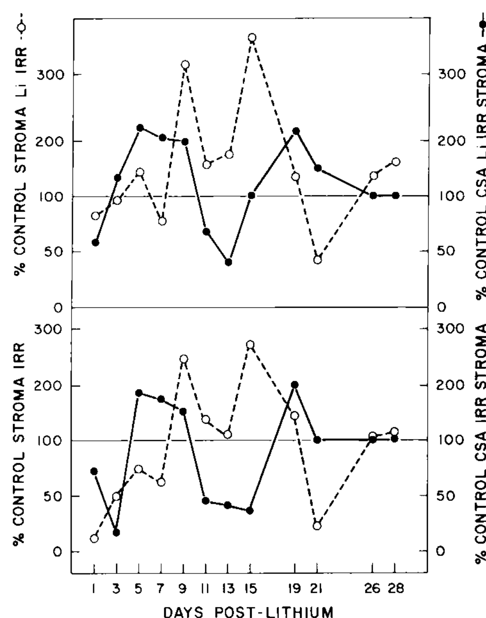

Fig. 3. The effect of lithium on the number of stromal colonies (○) derived from spleens taken from animals administered irradiation (2 Gy) and lithium (top) compared with those animals receiving irradiation only (bottom) and their ability to support normal non-adherent cell derived CFU-GM (●). Values expressed are the means taken from triplicate studies expressed as per cent of control.

Table

Stromal colonies harvested from the bone marrow (±SEM, stromal colonies/2×10^6 marrow cells) of normal mice administered lithium stained for cytochemical identification of granulocytes and monocytes/macrophages

Days after lithium	Group	Acetyl-cholin-esterase	Hema-toxylin	α-naphthyl	Naph-thol
1	Control*	0	37±2	37±2	0
	Lithium	0	56±5	51±1	4±2
2	Control	0	34±2	37±1	0
	Lithium	0	63±4	60±2	5±1
5	Control	0	37±5	35±2	0
	Lithium	0	96±6	90±4	13±2
7	Control	0	34±3	37±2	0
	Lithium	0	65±6	53±6	15±3
9	Control	0	33±4	36±2	0
	Lithium	0	53±5	42±3	17±1
12	Control	0	35±3	34±3	0
	Lithium	0	37±2	38±3	17±1
15	Control	0	33±1	37±2	0
	Lithium	0	34±3	35±1	8±1

* Phosphate buffered saline.

poietic stem cells and their immediate environment, known as the hematopoietic inductive microenvironment (20). The proper interaction between these two components is obligatory for the normal maintenance of steady-state hematopoiesis because without such cooperation, the normal proliferation and differentiation of stem cells is restricted.

The importance of the hematopoietic inductive microenvironment or stroma in the establishment of hematopoiesis has long been recognized. When hematopoietic tissue is implanted in ectopic sites, the tissue first reconstituted is that of the stroma (18, 19). It has also become known that when a specific hematopoietic organ's stroma becomes unsuitable for continued hematopoiesis, stromal cells migrate to other suitable organ sites to resume proliferation (15).

The administration of high dose irradiation and the subsequent failure for permanent hematopoietic reconstitution of these damaged sites has been demonstrated to occur because of permanent damage to the hematopoietic stroma (13). It is this type of damage that usually results in the development of complete aplasia seen after administration of high dose irradiation.

With the recent developments in in vitro technologies it has now become possible to study the hematopoietic stroma because stromal cells can and do form colonies. These colonies are usually comprised of fibroblastoid cells and can be maintained as cell lines (5, 6). With the development of these in vitro derived stromal colony assays, investigators can now examine the effects of various agents on their ability to interact with, assess the damage to or accelerate the recovery of the hematopoietic microenvironment.

Along these lines, we have previously demonstrated that lithium effectively accelerates the recovery of granulopoiesis after subacute radiation injury (10). Lithium generated an elevated recovery not only in the number of bone marrow and splenic derived granulocyte-macrophage colony forming stem cells (CFU-GM), but it also reduced the periods of neutropenia that are commonly associated with radiation induced injury to the hematopoietic system.

In this report we have further explored the capacity of lithium to enhance granulopoietic recovery by first measuring the ability of lithium to increase the number of both bone marrow and spleen derived stromal colonies in the steady-state, in addition to measuring the ability of lithium to influence the recovery of the stromal population after the administration of sub-lethal irradiation (2 Gy). Lithium was effective in increasing the number of stromal colonies derived from not only normal bone marrow and spleen but also from these tissues after radiation induced injury. These results demonstrate that lithium effectively can increase the number of stromal derived colony forming stem cells obtained from either bone marrow or spleen.

More importantly, these studies have demonstrated that the stroma can and does support the growth of hematopoietic progenitor cells, in this case, CFU-GM after radiation induced injury. It has been demonstrated by a number of investigators (1, 22) that stromal cells do produce colony stimulating factor (GM-CSF) which can support the growth and maintenance in vitro of CFU-GM. Here we provide further evidence that not only can stromal cells support CFU-GM derived colonies but such an ability is increased in the presence of lithium. Lithium has been demonstrated to increase the production of GM-CSF from a wide variety of cells (12, 16); therefore, lithium may have also increased the production of GM-CSF by stromal cells cultured in vitro from bone marrow and spleen cells harvested from animals that had been irradiated. Other mechanisms, however, could explain this lithium-induced response: 1) an altered sensitivity by CFU-GM to already existing levels of GM-CSF and 2) mediation via an accelerated recovery of the stromal cells due to a proliferative effect. Lithium has been demonstrated to increase the cell cycle kinetics of CFU-GM in vitro (9), to increase recruitment of progenitors into the megakaryocyte lineage (3) and by inducing a significant increase in the percentage of cells in S, G_2 and M phase from colonies cultured from human bone marrow in the presence of lithium (4).

In addition, lithium appeared to accelerate the presence of cells of the granulocyte lineage and those of the monocyte/macrophage type as measured by the presence of the granulocyte enzyme marker naphthol AS-D chloroacetate esterase when compared with the presence of the enzyme marker for monocyte/macrophage identification, α-naphthyl acetate esterase. These results could be interpreted to suggest that lithium not only influences the proliferation of such committed stem cells, but it may also influence the pattern of stem cell differentiation into one cell type or another. Further investigations

are required to more clearly define the relationships between the recovery of granulopoiesis, lithium and the hematopoietic stroma.

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