CONCISE REPORT

Suppression and Potentiation of Mouse Hematopoietic Progenitor Cell Proliferation by Ouabain

By Jerry L. Spivak, John Misiti, Robert Stuart, Saul J. Sharkis, and Lyle L. Sensenbrenner

Clonal assays for CFU-S, CFU-C, BFU-E, and CFU-E were employed to evaluate the effect of ouabain on the proliferation of mouse hematopoietic progenitor cells. Preincubation of bone marrow cells with ouabain at concentrations of 10^{-8} - 10^{-12} M suppressed the proliferation of CFU-S as measured by the spleen colony assay. At 10^{-4}M ouabain, spleen colony formation was inhibited by more than 95%. When included in soft agar cultures of bone marrow cells, ouabain suppressed the proliferation of CFU-C in a complex fashion. At 10^{-4}M ouabain, colony formation was suppressed by 70%, while at 10^{-4}M ouabain, CFU-C proliferation was normal. At 10^{-6}M ouabain, however, the number of colonies formed was only 70% of normal, and complete recovery was not obtained at 10^{-12}M ouabain. The number of colonies formed by BFU-E and CFU-E in plasma clot cultures was also diminished in the presence of 10^{-4}M ouabain. In contrast to its effect on CFU-S and CFU-C, however, 10^{-4}M ouabain enhanced BFU-E colony formation by 200% and CFU-E colony formation by 35%; at 10^{-12}M ouabain, CFU-E proliferation was unaffected by the drug. These data indicate that mouse CFU-S, CFU-C, BFU-E, and CFU-E require a functional membrane-bound Na^+K^+ATPase for proliferation; that these progenitor cells differ in their response to ouabain and finally, that ouabain at concentrations below that (10^{-4}M) considered necessary to inhibit membrane-bound Na^+K^+ATPase can influence the proliferation of mouse hematopoietic progenitor cells.

The mechanisms by which hematopoietic progenitor cells are induced to proliferate and differentiate are not understood. Less than 10% of pluriptotent hematopoietic stem cells (CFU-S) are in cell cycle under normal circumstances, while at least 20% of early erythroid progenitor cells (BFU-E) and 70% of late erythroid progenitor cells (CFU-E) are actively proliferating; the proportion of cycling granulocyte progenitor cells (CFU-C) is approximately 35%. Particular glycoproteins, erythropoietin, and colony-stimulating factor provide the stimulus for erythroid and granulocytic proliferation and differentiation, respectively, but no specific trophic agent has been identified for the pluripotent hematopoietic stem cell, nor is it clear how agents capable of triggering the proliferation and differentiation of hematopoietic progenitor cells effect these processes.

There is considerable evidence indicating that intracellular potassium concentration influences macromolecular synthesis and cell division. Protein synthesis is impaired when cultured cells are incubated in a medium low in potassium. Furthermore, ouabain (Strophanthin G) at concentrations (10^{-4}M - 10^{-7}M) that inhibit membrane-bound Na^+K^+ATPase blocks mitogen-induced lymphocyte transformation and the in vitro proliferation of lymphoblasts, fibroblasts, and Ehrlich ascites cells. Increasing the potassium concentration of the medium reverses the effect of ouabain and restores cell proliferation. In some tissues, however, inhibition of membrane-bound Na^+K^+ATPase serves to trigger proliferation and differentiation, since ouabain induces DNA synthesis and cell division in spinal cord neurons and differentiation of Friend erythroleukemic cells.

The possibility that variations in transmembrane monovalent cation flux might be responsible for the differences in proliferative activity of hematopoietic progenitor cells or serve as a stimulus for induction of proliferation and differentiation has not been investigated. To determine the influence of alterations in transmembrane cation flux on the proliferation of hematopoietic progenitor cells, we examined the effect of ouabain on clonal assays for mouse CFU-S, CFU-C, BFU-E, and CFU-E.

MATERIALS AND METHODS

Bone marrow cells from B6D2F, female mice weighing 20-25 g (Cumberland View Farms, Cumberland, Tenn.) were used for assay of CFU-S and CFU-E; for assay of BFU-E and CFU-E, marrow cells were obtained from either the B6D2F, mice or Swiss-Webster female mice weighing 20-25 g (Buckbeeberg Lab Animals, Tomkins Cove, N.Y.). CFU-S were assayed according to the method of Till and McCulloch. Before assay, the marrow cells were incubated in tissue culture medium with selected concentrations of ouabain at 37°C for 30 min. Lethally irradiated syngeneic recipients were injected by the tail vein with 10^7 treated or untreated marrow cells, and the number of macroscopic spleen colonies was determined 7 days later. Five mice were employed for each experimental point. An un.injected group of irradiated animals was included in each experiment to provide the stimulus for induction of proliferation and differentiation.
control for endogenous spleen colony formation; no endogenous colonies were observed in three separate experiments.

CFU-C were assayed by culturing $5 \times 10^5$ marrow cells in soft agar in the presence of lung-conditioned medium for 7 days. Selected concentrations of ouabain were added to the cultures at the time the cells were plated. Granulocyte-macrophage colonies containing more than 50 cells were counted with a dissecting microscope. Four separate plates were examined for each experimental point.

BFU-E and CFU-E were assayed in plasma clot cultures. For BFU-E, $5 \times 10^5$ marrow cells were cultured in plasma clots in the presence of selected concentrations of ouabain and 2.0 U/ml of erythropoietin (Sheep Plasma Step III, 7 U/mg protein, Connaught Laboratories, Toronto, Canada). For CFU-E, $10^6$ marrow cells were cultured in the presence of ouabain and 0.1 U/ml of sheep erythropoietin. After 48 hr for CFU-E and 7 days for BFU-E, the clots were fixed with glutaraldehyde and stained with benzidine and hematoxylin. Under light microscopy, all colonies containing 8 or more benzidine-positive nucleated cells were scored as CFU-E. Aggregates containing multiple distinct or confluent colonies of benzidine-positive nucleated cells were scored as BFU-E. Three to six clots were examined for each experimental point. Three to four separate experiments were performed with each class of progenitor cell, and Student's $t$ test was used for analysis of the significance of differences.

Ouabain was obtained from either Sigma Chemical Co. (St. Louis, Mo.) or Calbiochem-Behring Corp., American Hoechst Corp., (San Diego, Calif.). Different lots of ouabain from these sources gave identical results.

### RESULTS

Incubation of mouse bone marrow cells with ouabain at concentrations from $10^{-6}$ to $10^{-12}$ M for 30 min before injection into lethally irradiated recipients resulted in a profound reduction in the ability of CFU-S to form spleen colonies (Table 1). Inhibition was maximal at $10^{-9}$ M ouabain, and recovery was not complete at a concentration of $10^{-12}$ M ouabain.

Ouabain inhibited the proliferation of CFU-C in a complex fashion (Table 1). At $10^{-4}$ M ouabain, there was a 70% reduction in CFU-C, while at $10^{-6}$ M, CFU-C proliferation was normal. At $10^{-9}$ M colony formation was again inhibited by 30%, and recovery was not complete at $10^{-10}$ M ouabain.

The effect of ouabain on BFU-E and CFU-E proliferation differed from its effect on CFU-S and CFU-C.

### Table 1. Effect of Ouabain On CFU-S and CFU-C Proliferation

<table>
<thead>
<tr>
<th>Ouabain Concentration</th>
<th>BFU-S/10^5 Cells*</th>
<th>CFU-C/5 x 10^5 Cells*</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>21.7 ± 1.7</td>
<td>99.5 ± 0.6</td>
</tr>
<tr>
<td>$10^{-4}$M</td>
<td>ND†</td>
<td>26.5 ± 1.0‡</td>
</tr>
<tr>
<td>$10^{-6}$M</td>
<td>13.0 ± 3.0§</td>
<td>85.8 ± 2.5</td>
</tr>
<tr>
<td>$10^{-8}$M</td>
<td>0.25 ± 0.25$\ddag$</td>
<td>67.0 ± 0.1∥</td>
</tr>
<tr>
<td>$10^{-10}$M</td>
<td>11.2 ± 3.9§</td>
<td>70.5 ± 4.0</td>
</tr>
</tbody>
</table>

*Mean ± SEM.
†No data were obtained at $10^{-4}$ M ouabain.
‡Significantly different from control ($p < 0.0005$).
§Significantly different from control ($p < 0.02$).
∥Significantly different from control ($p < 0.002$).

Although BFU-E and CFU-E proliferation were markedly inhibited at $10^{-4}$ M ouabain, lower concentrations enhanced their proliferation (Table 2). At $10^{-7}$ to $10^{-12}$ M ouabain, there was a 36%–200% increase in the number of BFU-E. A 15%–35% increment in the number of CFU-E was observed at concentrations of ouabain between $10^{-6}$ M and $10^{-10}$ M. At $10^{-12}$ M ouabain, proliferation of CFU-E was unaffected by the drug. Similar results were obtained when marrow cells from Swiss-Webster mice were used instead of B6D2F1 cells. In the absence of erythropoietin, CFU-E proliferation was not stimulated by ouabain (data not shown).

### Table 2. Effect of Ouabain On BFU-E and CFU-E Proliferation

<table>
<thead>
<tr>
<th>Ouabain Concentration</th>
<th>BFU-E/5 x 10^5 Cells*</th>
<th>CFU-E/10^5 Cells*</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>6.3 ± 0.3</td>
<td>221.0 ± 8.0</td>
</tr>
<tr>
<td>$10^{-7}$M</td>
<td>1.0 ± 1.1</td>
<td>12.5 ± 2.3</td>
</tr>
<tr>
<td>$10^{-6}$M</td>
<td>6.6 ± 0.3</td>
<td>253.6 ± 4.9§</td>
</tr>
<tr>
<td>$10^{-5}$M</td>
<td>8.6 ± 0.3‡</td>
<td>255.6 ± 9.5§</td>
</tr>
<tr>
<td>$10^{-4}$M</td>
<td>12.0 ± 1.4‡</td>
<td>282.6 ± 27.4§</td>
</tr>
<tr>
<td>$10^{-3}$M</td>
<td>13.0 ± 1.0‡</td>
<td>297.7 ± 14.6§</td>
</tr>
<tr>
<td>$10^{-2}$M</td>
<td>10.5 ± 2.1§</td>
<td>260.5 ± 16.7§</td>
</tr>
<tr>
<td>$10^{-1}$M</td>
<td>11.2 ± 1.9∥</td>
<td>227.0 ± 3.1</td>
</tr>
</tbody>
</table>

*Mean ± SEM.
†Significantly different from control ($p < 0.002$).
‡Significantly different from control ($p < 0.01$).
§Significantly different from control ($p < 0.05$).
∥Significantly different from control ($p < 0.02$).

DISCUSSION

Three conclusions can be drawn from these data. First, the four classes of mouse hematopoietic progenitor cells examined require a functional membrane-bound Na'K'ATPase for proliferation. Second, the four classes of progenitor cells differ in their response to ouabain. Third, ouabain at concentrations below that ($10^{-6}$ M) considered necessary to inhibit membrane-bound Na'K'ATPase in rodents influences the proliferation of mouse hematopoietic progenitor cells.

Rodent cells are generally less sensitive than the cells of other species to ouabain and concentrations of the drug above $10^{-6}$ M are required to inhibit membrane-bound Na'K'ATPase. In our studies, hematopoietic colony formation was affected by ouabain over a wide concentration range, but inhibition of colony formation was uniformly obtained only at concentrations of $10^{-6}$ M or greater. This suggests that the inhibition was due to a ouabain-induced reduction in Na'K'ATPase activity.

At other concentrations of the drug, the hematopoietic progenitor cells were affected differently. Substantial inhibition of spleen colony formation was produced by $10^{-8}$ M ouabain; CFU-C proliferation was also inhibited at this concentration but to a lesser

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extent. In contrast, at $10^{-9}M$ ouabain, the proliferation of BFU-E and CFU-E was enhanced. Kinetic studies have demonstrated that BFU-E and CFU-C have a closer precursor–progeny relationship with CFU-S than do CFU-E.19,20 Our data, however, indicate that inspite of this proximity, BFU-E respond to ouabain differently than CFU-S.

Inhibition of CFU-S and CFU-C proliferation and enhancement of BFU-E and CFU-E proliferation by $10^{-9} M$ ouabain is a unique observation. At this concentration, ouabain stimulates Na$^+$ K$^+$ ATPase activity in guinea pig atria21 and microsomal preparations of rabbit brain and chicken kidneys.22 In view of the insensitivity of rodent cells to ouabain, it is unlikely that activation of Na$^+$ K$^+$ ATPase accounts for the results we obtained.

Ouabain consists of a steroid nucleus to which are attached a lactone ring and a rhamnose moiety. The effects of the drug at low concentrations, therefore, could be due to its steroidal properties. The influence of a variety of steroids on hematopoietic progenitor cell proliferation at concentrations of $10^{-7}M$ or less is well documented.4,23,24

Hematopoietic progenitor cells are also sensitive to $\beta$-adrenergic agonists.6 Since ouabain can stimulate cAMP formation,26 the glycoside could have influenced the proliferation of hematopoietic cells in that fashion.

Without direct measurements of cation flux, ouabain binding sites, intracellular ouabain concentration, and cAMP levels, the mechanism by which ouabain acts remains speculative. Owing to the cellular heterogeneity of the marrow, these measurements must await the development of techniques for purifying the cell populations involved. Nevertheless, the differential effects of ouabain on the proliferation of hematopoietic progenitor cells establishes the drug as a useful agent for cell kinetic studies.

REFERENCES


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