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CALCIUM ION-DEPENDENT PROLIFERATION OF L1210 CELLS IN CULTURE

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Maximum growth of L1210 cells in culture required the presence of free extracellular calcium ions. Reducing the free extracellular calcium ion concentration with EGTA served to decrease the growth rate of the cells. The decrease in cell growth was not due to cell death but rather due to the "pile-up" of the L1210 cells in the GO/G1 phase of the cell cycle. With the readdition of excess calcium ions, there was a lag period of 3 to 6 hours before the L1210 cells initiated DNA synthesis or transited from the GO/G1 phase to S-phase. Cells enriched for S and G2/M phases by elutriation and which were incubated in EGTA-containing culture medium, continued through the cell cycle and were blocked in GO/G1. These data indicate that the proliferation of L1210 cells in culture requires a calcium ion-dependent process to allow movement from the GO/G1 to S-phase of the cell cycle.

The processes which are required for cell proliferation are highly regulated and ordered and depend on the response to the appropriate external signals such as hormones, growth factors and calcium ions. Tumor cells, in most cases, show a loss or relaxation of these growth control parameters. A definite but undefined role for both extracellular and intracellular calcium ions has been demonstrated in many different types of studies (1-4). It has been reported that neoplastic cells do not have the same requirement for extracellular calcium ions as do normal cells (5, 6). However, at least some cancer cell lines were shown to require calcium ions for cell proliferation (7).

In the studies presented here, we show that highly malignant leukemia L1210 cells, which have a doubling time of 10-12 hours in

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culture, show a marked dependency on the free extracellular calcium ions for proliferation.

METHODS AND MATERIALS

Growth of L1210 Cells: L1210 cells were grown in RPMI 1640 culture medium supplemented with 10% horse serum, sodium bicarbonate (2 g/l) and gentamicin sulfate (50 mg/l). The cells were incubated at 37° C in 90% air/10% CO_2 in a humidified chamber. The L1210 cells were obtained from the American Tissue Type Collection and were checked regularly for mycoplasma contamination using the BRL Mycotect test.

In the experiments with EGTA, EGTA was added to the cells in culture at a final concentration of 2.2 mM.

Cell Cycle Analyses: L1210 cells were stained with propidium iodide using the method of Krishan (8). L1210 cells (1×10^6) were collected by centrifugation. The culture medium was removed and to the cell pellet was added sodium citrate (0.03M, 0.050 ml) and water (0.445 ml). The cells were resuspended by vigorous vortex mixing. A filtered propidium iodide solution (5 mg/ml in H_2O, 0.005 ml) was added and the cells were incubated on ice for 20 min. in the dark. Ribonuclease (4 u/ml) was added and the cells incubated for 30 min. at room temperature in the dark. The cells were then kept overnight at 5° C before the flow cytometry runs were made. The analyses were carried out on a Coulter EPICS C flow cytometer.

Thymidine Incorporation into DNA: The L1210 cells in culture were incubated with [^{3}H]dThd (50 Ci/mmole, 5 μCi/flask) for 30 min. at 37° C. The cells were harvested by centrifugation, washed with phosphate-buffered saline and recentrifuged. To the cell pellet, albumin (0.5 ml, 5 mg/ml) and perchloric acid (0.5 ml, 12%) were added and the cells disrupted with a loose-fitting motor-driven teflon pestle. After centrifugation, the supernatant fluid was removed and the pellet reextracted with perchloric acid (1 ml, 6%) and recentrifuged. The pellet was solubilized in NaOH (1 ml, 0.25 M) and an aliquot taken for radioactivity determinations.

RESULTS AND DISCUSSION

When L1210 cells are grown in tissue culture in RPMI 1640 medium supplemented with 10% horse serum, the extracellular concentration of free calcium ions is approximately 0.7 mM. When EGTA (2.2 mM) was added to the culture medium, there was little cell growth as shown in Fig. 1. When additional calcium ions (2.2 mM) were added at the same time as the EGTA, the cells grew to the same extent as the untreated controls indicating that EGTA, per se, was not toxic. At a concentration of calcium ions of 1.1 mM, there was a pronounced lag in cell growth, especially seen at day-3. There was essentially no cell growth when only 0.22 mM calcium ions were added (data not shown). The addition of calcium ions

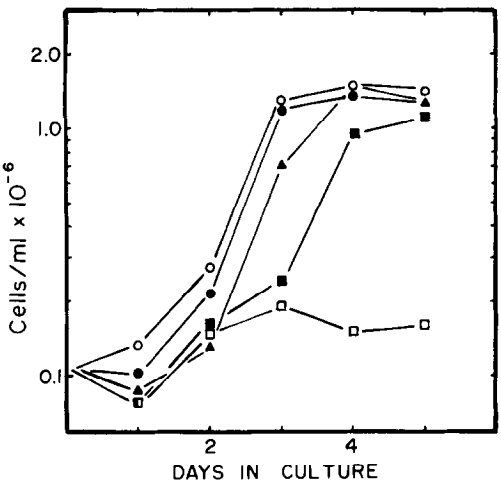

Fig. 1. L1210 Cell Growth in the Presence of EGTA and Calcium Ions. L1210 cells were grown under normal conditions (○); in the presence of EGTA, 2.2 mM (□); in the presence of EGTA and calcium ions added simultaneously, 2.2 mM each (●); in the presence of EGTA, when the calcium ions, 2.2 mM, were added 24 hrs after the addition of EGTA (△); or in the presence of EGTA, when the calcium ions, 1.1 mM, were added 24 hrs after the addition of EGTA (■).

to the culture medium 24 hrs after the addition of the EGTA resulted in the resumption of cell proliferation after a lag period of about 24 hrs.

Pulse labeling of the DNA with [^{3}H]dThd was decreased by 99% in the cells treated with EGTA for 24 hrs. Cell cycle analysis by flow cytometry indicated that the cells were primarily in GO/G1 phase of the cell cycle. These data were summarized in Table 1. The decrease in cell growth in the absence of extracellular calcium ions was not due to cell death but rather due to the "pile-up" of the L1210 cells in the GO/G1 phase of the cell cycle.

The EGTA-induced effect was time dependent, requiring 24 hours to reach the maximum decrease in [^{3}H]dThd incorporation into DNA. Preincubation of the culture medium with EGTA, prior to use with the cell cultures did not alter the required time (24 hours) for bringing about the maximal effect.

The reversal of the EGTA effect by calcium ions was time-dependent. As seen in Fig. 2, there was a lag of at least 3 hours

Table 1. Effect of Calcium Ion Deprivation on Thymidine Incorporation into DNA and Cell Cycle of L1210 Cells

Treatment	DNA cpm/10^6 cells	% Cell Cycle G0/G1	S	G2/M
None	26,600	45	39	16
EGTA, 2.2 mM	400	80	17	3
EGTA plus Ca^{+2}, 2.2 mM, t=0	22,600	49	37	14

before a response, such as increased [^{3}H]dThd incorporation into DNA or transit from the GO/G1 phase was observed. In comparison, the GO/G1 population enriched by centrifugal elutriation did not show the corresponding lag with respect to [^{3}H]dThd incorporation into DNA or transit from GO/G1 (data not shown). This would indicate that the EGTA-induced GO/G1 L1210 cell population was blocked at an earlier point in the cycle, perhaps, GO.

The reversal of the EGTA effect was specific for calcium ions;

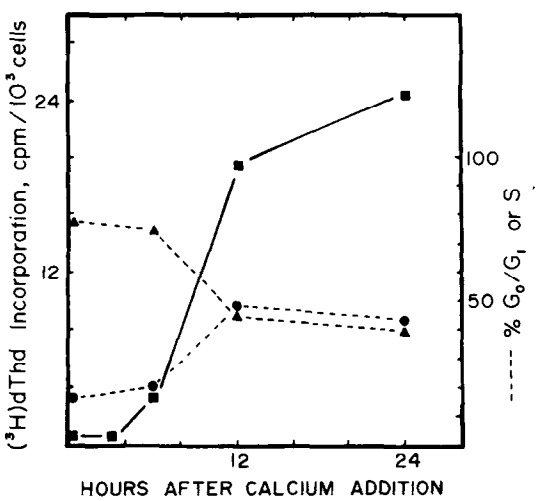

Fig. 2. Time Course for Calcium Ion-induced Effects in L1210 Cells. L1210 cells were incubated in the presence of EGTA, 2.2 mM, for 24 hrs. Calcium ions, 2.2 mM, were added and the incorporation of [^{3}H]dThd (■) into DNA and cell cycle analyses were determined as a function of time after calcium ion addition. G0/G1 cells (▲) and S phase cells (●) were determined by the method of Krishan (8) using a Coulter EPICS C cytofluorometer.

neither magnesium nor ferrous ions reversed the EGTA-induced effect.

After the readdition of calcium ions to the EGTA treated cells, the presence of calcium ions was required throughout the entire period to reach maximal response. L1210 cells which had been incubated with calcium ions for only 8 hours, and then resuspended in culture medium with EGTA for the next 16 hours, only reached 31% (as measured by dThd incorporation into DNA) of the control cells which had been incubated in the presence of the additional calcium ions for the full 24 hours.

Protein synthesis was required for the calcium ion induced effects. When cycloheximide (10 μg/ml) was added at either t=0 or t=6 hr after calcium ion addition to the EGTA-treated cells, there was no increase in [^{3}H]dThd incorporation into DNA. As shown earlier (Table 1 and Fig. 2), L1210 cells, treated with EGTA to remove free extracellular calcium ions, accumulate in the GO/G1 phase of the cell cycle. When L1210 cells were enriched for GO/G1, S and G2/M phase cells by centrifugal elutriation, and put into culture medium containing EGTA and incubated for 24 hrs, it was found that the three populations of cells accumulated essentially to the same extent in GO/G1 phase (60-73%). Thymidine incorporation into DNA followed the same pattern. These data are shown in Table 2. In the GO/G1, S or G2/M enriched cell populations cultured in the presence of EGTA for 24 hrs, [^{3}H]dThd incorporation into DNA was only 1 to 3% of the controls. In the cell populations grown in the presence of EGTA and calcium ions (both at 2.2 mM), [^{3}H]dThd incorporation into DNA was 91 to 93% of the control values and the distribution of cells in the phases of the cell cycle were very similar to the controls. From the data presented in Table 2, it is clear that the reduction of free extracellular calcium ions with EGTA resulted in a rather specific

Table 2. Effect of Calcium Ion Deprivation on Cell Cycle Phase Enriched L1210 Cells

Cell enrichment	Treatment	DNA $cpm/10^6$ cells	% Cell Cycle G0/G1	% Cell Cycle S
G0/G1 (83%)	none	97,700	39	41
	EGTA	3,000	63	23
	EGTA, Ca^{+2}	89,100	36	45
S (55%)	none	82,200	36	49
	EGTA	1,100	73	19
	EGTA, Ca^{+2}	76,500	38	47
G2/M (41%)	none	57,900	35	42
	EGTA	2,000	60	28
	EGTA, Ca^{+2}	52,700	34	44

L1210 cells were enriched for cell cycle fractions by centrifugal elutriation in the %'s shown in the parentheses.

inhibition of some process or processes required for the transition from G0/G1 to S. The S phase and G2/M phase cells when cultured in the presence of EGTA continued through their cell cycles and were then primarily blocked in G0/G1.

These studies demonstrate that the leukemia L1210 cells, a highly malignant cell line, have an extracellular requirement for calcium ions which is as great as that reported for normal cells (1-6). Future studies will detail the biochemical events which follow the reintroduction of free extracellular calcium ions to those cells which have been blocked in G0/G1.

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