TRANSITION METAL IONS INDUCE CELL GROWTH IN NRK CELLS SYNCHRONIZED IN G_1 BY PICOLINIC ACID

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SUMMARY The G_1 arrest induced in NRK cells by picolinic acid could be prevented by addition of Fe^{3+}, Zn^{2+} or Co^{2+} to the tissue culture media. Ca^{2+}, Mg^{2+}, Mn^{2+}, Sr^{2+} or Ba^{2+} were ineffective. Complete and synchronous reversal of the G_1 block, however, was achieved by Fe^{3+} at lower concentration from that of Zn^{2+}. Co^{2+} reversed the block but cells divided asynchronously. Thymidine incorporation, mitotic index and relative DNA content per cell, verified that G_1 arrested cells proceeded through the cell cycle after addition of Fe^{3+} or Zn^{2+}. These observations afford a valuable model system for elucidating the biochemical events that occur between addition of a defined proliferative signal and stimulation of DNA synthesis in G_1 arrested cells.

To test an hypothesis concerning the nature of growth control (J. A. Fernandez-Pol, unpublished) picolinic acid was tested and found to be a powerful tool to study cell growth regulation (1-3). Picolinic acid reversibly arrested NRK (normal rat kidney) cell growth in the $G_1(G_0)$ phase of the cell cycle (2-3). However, the block in transformed cells was dependent upon the transforming virus (2). The mechanism of this inhibition and how picolinic acid interacts with a putative growth control mechanism which may be selectively altered by transformation is not known (2). Picolinic acid is of further interest because it is a naturally occurring compound, but it has no known functions (2). Here I present evidence that picolinic acid may primarily act through its chelating ability, depriving NRK cells from an essential ion which is required for the initiation of DNA synthesis. Most likely, the ions involved in the mechanism of action of picolinic acid are Fe^{3+} and/or Zn^{2+}.

MATERIALS AND METHODS

Materials Alpha-picolinic acid was obtained from Sigma (St. Louis, Mo.) $FeCl_3$, $ZnCl_2$, $MnCl_2$, $MgCl_2$, $CoCl_2$, $BaCl_2$, $CaCl_2$ and $SrCl_2$ were obtained from Fisher Scientific (Fairlawn, N.J.). [^{3}H] thymidine was obtained from Amersham-

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Searle. Tissue culture media was purchased from Microbiological Associates (Bethesda, Maryland). Ca^{2+} Ionophore A23187 was generously provided by R.J. Hosley from Eli Lilly (Indianapolis, Indiana).

Methods Cells were grown in Dulbecco-Vogt modified Eagle's medium containing 10% (V/V) calf serum (Colorado Serum Co.) as previously described (3). NRK cells (3) were obtained from E. Scolnick (NIH). Clone 5W isolated by D. Wallach (NIH) was used in this study. Cells were planted at 1.5×10^5 cells/dish in 20 cm^2 plastic tissue culture dishes (Costar). Forty-eight hours later, media were changed by new media without (control) or with (treated) 3 mM picolinic acid and/or ions. Cells were analyzed for DNA content by flow microfluorometry (FMF) using a cytofluorograph 4800A (Biophysics System, Inc., Baldwin Place, N.Y.) as previously described (2). Cells were counted in a Coulter counter. Thymidine incorporation was performed (2) with 0.05 µCi [^{3}H] thymidine per dish.

RESULTS

Effects of Ions on the Growth Inhibition Induced by Picolinic Acid It has been previously demonstrated that the growth inhibition was specific for picolinic acid (2). NRK cells treated for 48 hours with 3 mM picolinic acid ceased growth (Fig. 1) and were 90% synchronized in G_1 as determined by FMF analysis (2-3). To determine the effects of ions on cell growth and on the growth inhibition induced by picolinic acid, I tested several ions over a wide concentration range (0.001mM-3mM). Increased ion concentration in tissue culture media of control cultures either did not have any effect on the rate of cell growth or induced, in a dose dependent manner, growth inhibition (Fig. 1). Higher ion concentrations resulted in obvious toxicity which could be selectively antagonized by picolinic acid (Fig. 1).

Ca^{2+} and Mg^{2+} have previously been implicated in the control of cell growth (4-5). However, the inhibition of growth induced by picolinic acid appears not to be due to chelation of such metal ions because they did not antagonize picolinic acid effects. The addition of the Ca^{2+} Ionophore A23187 (6) at several different concentrations between 10^{-6} and 10^{-7}M failed to antagonize picolinic acid effects or to stimulate DNA synthesis in G_1 arrested cells. At higher concentrations it caused toxicity. These results are in agreement with results in SV40 transformed Balb 3T3 cells (2). Mn^{2+} was toxic and failed to activate the G_1 arrested cells at subtoxic levels (0.01 mM).

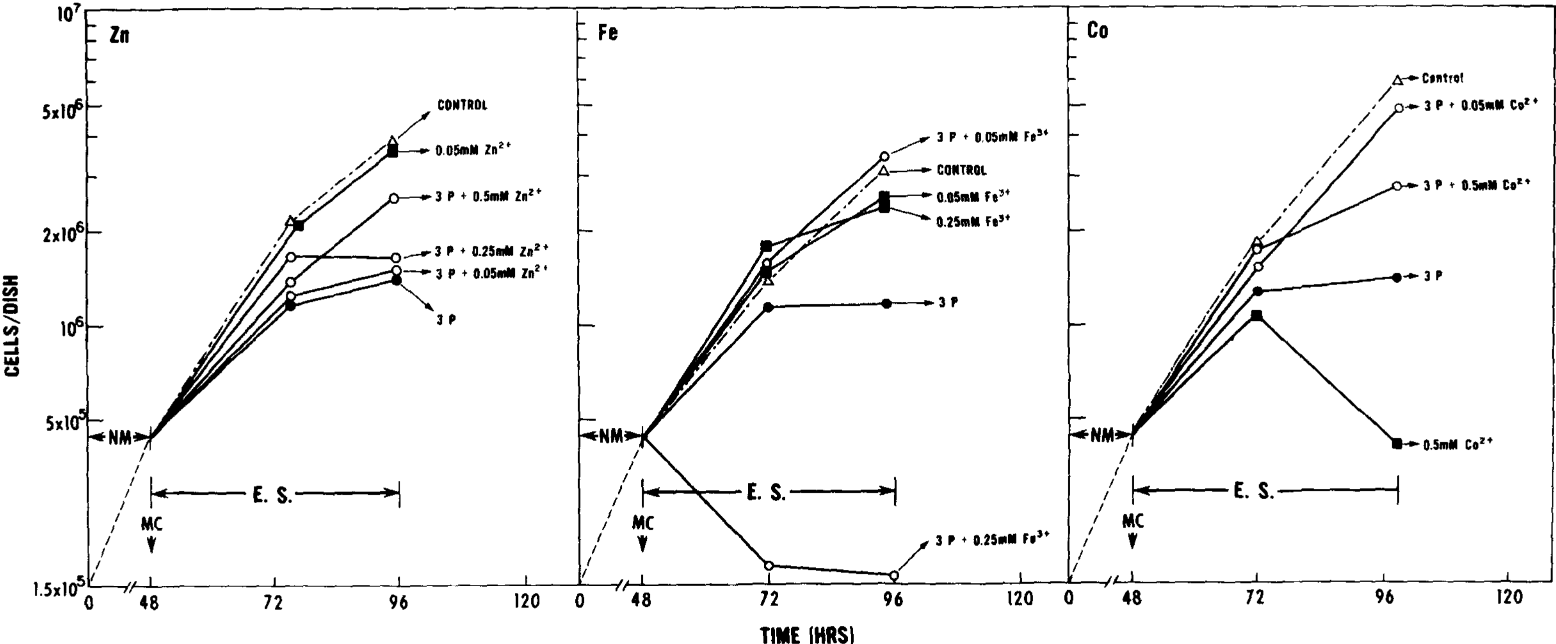

Fig. 1 Effects of Zn^{2+}, Fe^{3+}, or Co^{2+} on the growth inhibition induced by picolinic acid. In this and other experiments the reported concentrations do not include the contribution from ions contained in the serum present. Cells were planted at 1.5×10^5 cells/dish and 48 hours later the medium was removed and new media containing the indicated compounds were added. Cell counts were determined at the indicated times; each point is the average of duplicate measurements from five cultures. 3P: 3mM picolinic acid; NM: Normal medium; MC: Media change; ES: Experimental substances.

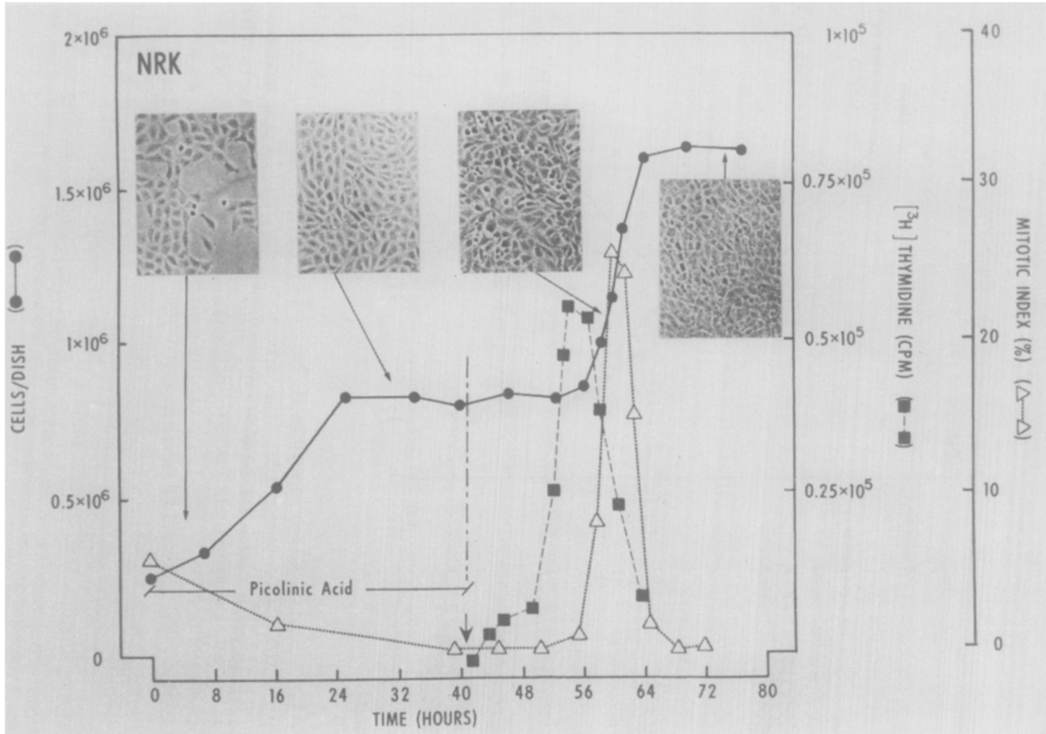

Fig. 2 Cell cycle analysis of NRK cells arrested in G_1 by picolinic acid and stimulated to proliferate by addition of Fe^{3+}. Cells were planted at 1.5 x 10^5 cells/dish. After 42 hr in picolinic acid, 0.05 mM Fe^{3+} were added (arrow). The cultures were observed periodically with phase contrast microscopy and the appearance of the cells at the indicated times are shown. Each cell count is the average of duplicate measurements from four cultures. The thymidine incorporation is the average from two cultures.

Sr^{2+} and Ba^{2+} were also ineffective. By contrast, prevention of the G_1 arrest was effectively obtained by addition of Fe^{3+}, Zn^{2+} or Co^{2+} to the tissue culture media (Fig. 1).

Induction of Growth in G_1 Arrested NRK Cells by Ions The progression through the cell cycle of NRK cells arrested in G_1 by picolinic acid was studied after addition of Co^{2+}, Fe^{2+} or Zn^{2+}. Co^{2+} reversed the G_1 block but cells began to divide asynchronously after 30 hours (data not shown). Therefore, the kinetics of the reversal suggests that picolinic acid does not exert its effects by chelating Co^{2+} (2). Addition of Fe^{3+} or Zn^{2+} to G_1 arrested

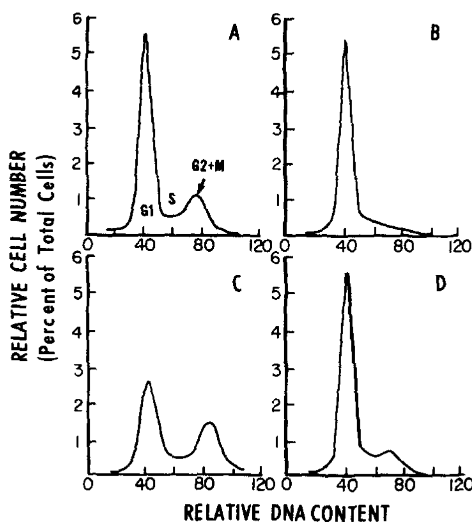

Fig. 3 Sequential DNA distribution profiles of NRK cells arrested in G_1 by picolinic acid and stimulated to proliferate by addition of Fe^{3+}. Cells were grown and treated as described in the text.

A. Control: Logarithmic growing cells.

B. Cells exposed to 3 mM picolinic acid for 42 hr.

C. Eighteen hr after Fe^{3+} addition, the movement through the cell cycle is shown by an increase in G_2 + M.

D. Twenty-four hr after addition of Fe^{3+}, and following the first wave of mitosis, essentially all cells are in G_1.

cells, however, stimulated synchronous growth, being Fe^{3+} effective at lower concentration than Zn^{2+} (data not shown). In order to confirm these results a cell cycle analysis was done using Fe^{3+} as the revertant agent. As shown in Figure 2 and 3B, NRK cells treated for 48 hours with 3 mM picolinic acid were predominantly in G_1. At this time, essentially no mitotic cells were observed. As shown in Figure 2, a wave of DNA synthesis began 12 hours after addition of 0.05 mM $FeCl_3$ to the tissue culture media, followed by a burst of mitotic activity and a doubling in cell number from 18-22 hours. To quantitate and further verify the progression of the cells through the cell cycle after Fe^{3+} addition, FMF analysis were done (Fig. 3). When Fe^{3+} was added, the number of cells in S and G_2 (Fig. 3C) increased and followed the first wave of mitosis, essentially all the cells were in G_1 (Fig. 3D). Thus, NRK cells arrested in G_1 by picolinic acid proceeded through the cell cycle after addition of Fe^{3+}.

These results are equivalent to removal of picolinic acid and addition of new media without picolinic acid (2).

DISCUSSION

Although cyclic AMP metabolism (3) and nicotinamide (1) have been implicated in the mechanism of action of picolinic acid, the results presented here indicate that the G_1 arrest induced by this agent in NRK cells may be primarily due to chelation of Zn^{2+} and/or Fe^{3+}.

Zn^{2+} and Fe^{3+} have been previously implicated in the regulation of DNA synthesis (7-8). The occurrence of Zn^{2+} in several enzymes associated with nucleic acid synthesis and speculation about possible molecular bases for Zn^{2+} requirements have been reviewed (9). Meager information exists about Fe^{3+} and cell growth in mammalian cells. In HeLa cells in culture, the iron-chelating agent desferrioxamine inhibited cellular iron uptake and halted DNA synthesis (8). The same agent caused cell death in cultured Novikoff hepatoma cells, an effect which could be reversed by addition of iron to the culture (10). Furthermore, the identification of transferrin as a lymphocyte growth promoter in human serum establishes an important role for iron in growth regulation (11). This suggests that picolinic acid could bind to and inactivate a specific protein-metal ion complex, such as transferrin, in a manner similar to the binding of nicotinic acid to leghemoglobin (12).

The present studies cannot allocate the specificity of picolinic acid to chelation of either Zn^{2+}, Fe^{3+} or both. However, they clearly do indicate that Fe^{3+} and/or Zn^{2+} deprivation exerts its effects preferentially in the G_1 phase of the cell cycle in NRK cells. These findings all support the concept that availability of Fe^{3+} and/or Zn^{2+} within the cell may play a central role in the coordinated control of events leading to initiation of DNA synthesis in $G_1(G_0)$ arrested cells. Additionally, these observations afford a new powerful tool for studying growth arrest and growth stimulation under controlled conditions by known agents.

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