Pyridoxine Resistance in a Rat Hepatoma Cell Line

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ABSTRACT

The natural vitamin, pyridoxine, in the millimolar range is toxic to cultured rat hepatoma cells. A pyridoxine-resistant Fu5-5 rat hepatoma cell line was established by a stepwise increase in the concentration of pyridoxine in the medium. The newly established cell line, referred to as clone 10 (CI.10), is resistant to killing by pyridoxine in concentrations up to 5 mM. Saturation kinetics for the uptake of $[^3]H$pyridoxine into Fu5-5 and CI.10 cells revealed that Fu5-5 cells take up 10 times more $[^3]H$pyridoxine than do CI.10 cells. Whereas the $V_{max}$ value for the uptake of $[^3]H$pyridoxine was the same for both cell lines, the apparent $K_m$ for the CI.10 cells was 12.5 μM compared to 0.71 μM for the Fu5-5 cells. However, intracellular levels of pyridoxal 5'-phosphate were 69% higher in CI.10 cells than in the parental line. The resistant line is neither a permeability mutant nor deficient in pyridoxal kinase. CI.10 cells contain 37% more adenosine 5'-triphosphate than do Fu5-5 cells and have a mitochondrial volume that is 50% greater than that of the parental line. In the absence of pyridoxine in the medium, CI.10 cells revert to parental type with respect to pyridoxine uptake but not with respect to resistance to killing. They also maintain an enlarged mitochondrial volume. Thus, increased mitochondrial volume may be related to the development of resistance to high levels of pyridoxine.

INTRODUCTION

We have demonstrated that high doses of vitamin B₆ when added to tissue culture medium prevented the proliferation of Fu5-5 rat hepatoma cells (5). Although the mechanism by which vitamin B₆ inhibited cell growth was not ascertained, experimentation did reveal that cells cultured for 6 hr in vitamin B₆-enriched medium took up and incorporated less $[^3]H$thymidine than did control cultures. Further studies indicated that the intracellular levels of ATP were elevated in cultures exposed to the vitamin for short periods of time. If cells were cultured in high-vitamin B₆-supplemented medium for more than 4 days, cell death occurred.

Since Fu5-5 rat hepatoma cells were unable to sustain growth in culture medium supplemented with high doses of vitamin B₆, studies to elucidate how the vitamin regulates cell growth were unsuccessful. Therefore, it seemed necessary to develop a cell line that was resistant to killing by high doses of vitamin B₆ but still transported the vitamin into the cell and metabolized it to the active vitamer, PLP.⁴

In the present communication, we report the establishment of a pyridoxine-resistant hepatoma cell line. The newly established cell line is shown to be resistant to killing by 5 mM pyridoxine-supplemented medium. Resistance appears to be the result of a mutation other than one in permeability of the membrane to pyridoxine or one in the pyridoxal kinase enzyme system. The mutation may involve some feature of mitochondrial development. The possibility of using the pyridoxine-resistant cell line to investigate the regulation of several key metabolite enzymes by PLP is discussed.

MATERIALS AND METHODS

Cell Culture Conditions

Eagle’s MEM (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% calf serum (M. A. Bioproducts, Walkersville, Md.), gentamicin (50 μg/ml; M. A. Bioproducts), and 2 mM glutamine were used for maintaining Fu5-5 rat hepatoma cells. Pyridoxine-resistant Fu5-5 rat hepatoma cells were maintained in the same medium but supplemented with a final concentration of 5 mM neutralized pyridoxine HCl (Sigma Chemical Co., St. Louis, Mo.). Cells were grown in Falcon T-75 sq cm flasks and maintained at 37° in a humidified atmosphere of 5% CO₂ in air.

Establishment of Pyridoxine-resistant Cell Line

Fu5-5 rat hepatoma cell line was derived from the H4-II-E-C3 line (4). Cells were plated in T-75 sq cm flasks and cultured for 2 weeks in growth medium supplemented with 0.5 mM neutralized pyridoxine. The pyridoxine concentration was then increased in increments of 0.5 mM every 2 weeks until a final concentration of 5 mM was reached. Since the increased concentrations of pyridoxine caused cell death (trypan blue exclusion test), the remaining viable cells, resistant to 5 mM pyridoxine, formed distinct colonies. These colonies were subsequently cloned in T-25 sq cm flasks and maintained in culture medium supplemented with 5 mM pyridoxine. Of the colonies cloned, one survived and was established in culture. This pyridoxine-resistant cell line is referred to as Fu5-5 PR clone 10 and is abbreviated as CI.10 elsewhere in this paper.


Pyridoxine resistant CI.10 and control Fu5-5 cells were cultured in serum-free MEM without pyridoxine (No. 78-0707; Grand Island Biological Co.) for 48 hr before use. At the start of the experiment, medium was aspirated off, and 10 ml of pyridoxine-free, serum-free MEM were added to each T-75 sq cm flask. To this medium was added $[^3]H$pyridoxine (specific activity, 1.5 Ci/mmol; Amer sham–Searle Corp.) to a final concentration of 1.33 μM. Flasks were incubated for various times. At the end of each time period, the medium was removed, and...
the cells were washed 3 times in PBS, and then dissolved in 3.0 ml of 1 N NaOH. For the determination of [3H]pyridoxine uptake as a function of its concentration, the protocol was the same as above. Radioactivity was measured in a liquid scintillation counter as described previously (20).

Determination of PL and PLP

The determination of the intracellular levels of both PL and PLP was carried out essentially by the procedure of Wada and Snell (19) with the following modifications. Cells grown for 48 hr in serum-free medium were trypsinized off the substrate (0.1% trypsin-0.02% EDTA in PBS without Ca²⁺ or Mg²⁺) and placed into 15-ml glass conical tubes. The cells were washed 3 times in PBS, and the final supernatant fraction was discarded. Cold 10% (w/v) trichloroacetic acid was added to the cell pellet to a final volume of 0.95 ml. The mixture was gently vortexed, kept on ice for 30 min, and centrifuged for 10 min in a Dynac table top centrifuge at maximum speed. The supernatant was removed, 0.05 ml of phenylhydrazine reagent was added to it (phenylhydrazine hydrochloride, 2 g dissolved in 100 ml of 36 N H₂SO₄) and the mixture was kept at 4°C for 30 min. This procedure allows for the determination of PLP in the presence of PL. For the measurement of PL, the reaction mixture was heated to 60°C for 20 min, cooled at room temperature, and assayed 10 min later. This procedure measures both PL and PLP concentrations. By subtracting the PLP measured at 4°C from that measured in the heated mixture (PL plus PLP), a value for the PL concentration can be obtained. All readings were made at 410 nm.

ATP Levels in Fu5-5, CI.10, and CI.10R Cells

Fu5-5, CI.10, and CI.10R cells were trypsinized off the substratum and placed into 15-ml conical tubes containing MEM. The cell suspension was centrifuged, and the supernatant fraction was discarded. To the pellet was added 1.0 ml of serum-free MEM, and a cell suspension was made by gently vortexing the mixture. Cell suspension (0.2 ml) was mixed with an equal volume of nucleotide releasing reagent (Lumac Systems, Inc., Titusville, Fla.), and the released ATP was then measured in a Luminometer M 1070 (Lumac Systems, Inc.) in the presence of the bioluminescence reagent LUMIT. LUMIT is a highly purified luciferin-luciferase reagent for sensitive measurement of ATP. ATP concentration is expressed as nmol/mg protein.

Protein Analysis

Protein concentration was estimated by the method of Lowry et al. (11).

Morphology and Stereology

TEM. Fu5-5 and CI.10 cells were prepared for TEM to determine if alterations in the mitochondrial population were correlated with the increased levels of ATP seen in CI.10 cells.

Cells were plated onto Aclar dishes (12), previously coated with rat tail tendon collagen (1), and cultured as described above. Prior to fixation, the cultures were washed 3 times with warm serum-free MEM and then fixed for 15 min at 37°C with 2% glutaraldehyde-0.1 M cacodylate containing 5% sucrose at pH 7.4. Fixation was continued at 4°C for 1.75 hr in fresh fixative. After fixation, the cultures were rinsed in 4 changes of cold buffer (0.1 M cacodylate containing 5% sucrose), postfixed in 1.33% OsO₄-0.067 M cacodylate with 5% sucrose for 1 hr, rinsed in buffer, stained en bloc with cold buffered uranyl acetate (6) for 1 hr at pH 6 in the dark, dehydrated in a series of graded ethanol, embedded in epoxy resin (17), and polymerized for 24 hr at 70°C. Areas (1 cm²) of the embedded cultures were cut out using a jeweler’s saw and were oriented parallel to the long axis of the cells by attaching them to a block previously polymerized with epoxy glue. Pale gold to silver thin sections were cut with a diamond knife and picked up on coated copper grids. These sections were stained with lead citrate for 2 min (16) or with uranyl acetate for 5 to 15 min followed by lead citrate and examined in a Phillips 300 electron microscope.

SEM. To detect if the surface of Fu5-5 and CI.10 cells differed in their topography, 5 samples of each cell line were examined after preparation for SEM. As for TEM, the cells were plated in Aclar dishes, allowed to grow for 24 hr, and then fixed as described above. After fixation, the cultures, still in Aclar dishes, were dried using absolute ethanol and liquid CO₂ in a Sorvall critical-point drying apparatus (DuPont Instruments-Sorvall Biomedical Div. DuPont Co., Wilmington, Del.) and attached to aluminum stubs with double-sided tape. They were then coated with a 15- to 20-nm layer of gold in a Denton Vacuum Desk-1 sputter coater (Denton Vacuum, Inc., Cherry Hill, N. J.) and examined in an ETEC Autoscan electron microscope at an accelerating voltage of 20 kV. Since the sample was disc shaped, 5 photographs were taken of each of its quadrants for each specimen. Polaroid PN-55 film was used for photographing the samples.

Stereology. Simple inspection of the TEMs suggested that CI.10 cells contained an increased mitochondrial compartment as compared to the parental cell line. Stereology was used to examine this possibility. The CI.10R line was analyzed in a similar way.

All of the samples examined were processed simultaneously for TEM. Four blocks of each category, Fu5-5, CI.10, and CI.10R, were chosen at random. To avoid counting the same cell more than once, only one thin section was selected from each block and examined. Each section was scanned using a nonrandom pattern, and the first 16 cells that met 2 previously established criteria (14, 15) were photographed. Each negative was enlarged photographically to 15,700 diameters. The fraction of the cell occupied by mitochondria was determined by a point-counting method (21) in which a transparent overlay bearing a grid pattern that consisted of 108 test points was superimposed over the photographic print. The points lying over mitochondria and those over the remaining cytoplasm and nuclei were tabulated. For each cell line, 64 cells were photographed and analyzed. Since nuclei were included in the fields, the data are expressed as percentage of cell volume (fractional cell volume). Data were analyzed with a one-way analysis of variance. The Newman-Keul test was used to locate significant differences among groups.

RESULTS

Effect of Pyridoxine on the Growth of Fu5-5 Rat Hepatoma Cells in Culture. Little is known of the growth characteristics of Fu5-5 rat hepatoma cells cultured in various vitamin B₆ concentrations. Therefore, a growth profile was constructed in which cell density was measured as a function of varying concentrations of pyridoxine in the medium. Results of this experiment are shown in Chart 1. Cells that had been cultured previously for 24 hr in MEM were switched to MEM containing various concentrations of pyridoxine. Cells cultured in the absence of pyridoxine showed no cell growth and in fact were dying. Cell growth was optimal at a pyridoxine concentration of 5 µM. Above this pyridoxine concentration, cell growth slowed; at a pyridoxine concentration of 5 to 10 µM, cell death occurred.

Establishment of a Pyridoxine-resistant Cell Line. Chart 1 indicates that high doses of pyridoxine severely retard cell growth and, at concentrations of 5 µM and above, cause cell death. Therefore, in an attempt to establish a cell line resistant to the killing effect of pyridoxine, a protocol was implemented whereby cells were exposed to increasing concentrations of pyridoxine over a prolonged period of time (see "Materials and Methods"). A stepwise increase in the concentration of pyridoxine in the medium was used. After exposure to the highest...
growth of Fu5-5 rat hepatoma cells in culture. Fu5-5 rat hepatoma cells were plated in T-75 sq cm flasks (1 x 10⁶ cells/flask) and cultured for 24 hr in MEM. After this incubation period, cells were changed into MEM containing various concentrations of pyridoxine and cultured for an additional 72 hr. Cell density was measured by counting cells in a hemocytometer in the presence of trypan blue. Results are the means of 3 T-75 sq cm flasks/point.

Chart 2. Cellular uptake of [³H]pyridoxine into Fu5-5 and Cl.10 cells. [³H]-Pyridoxine (final concentration, 1.33 μM) was added to cell cultures as described in “Materials and Methods” and incubated for various times. At the end of each time point, the medium was removed, and the cells were washed 3 times in PBS and then dissolved in 1 N NaOH. Each time point represents the mean value of 3 T-75 sq cm flasks.

Uptake of [³H]Pyridoxine into Fu5-5 and Cl.10 Cells. Initial experiments to elucidate the mechanism of resistance of Cl.10 cells to 5 mM pyridoxine were centered on the uptake of [³H]-pyridoxine into these cells. When both cell lines were analyzed for their ability to take up [³H]pyridoxine, Cl.10 cells concentrated only one-tenth as much [³H]pyridoxine after 60 min of incubation as did Fu5-5 cells (Chart 2). The resistant Cl.10 cells undoubtedly represent a selection of a preexisting mutant produced by the selective pressure of the high pyridoxine dose. At first, these cells appeared to be permeability mutants. A reversion of the permeability phenotype was tested by removing Cl.10 cells from the high pyridoxine-supplemented medium and placing them in a pyridoxine-free medium. Cells were then analyzed daily for their ability to take up the B⁶ vitamer. As shown in Chart 3, Cl.10 cells increased their daily uptake of [³H]pyridoxine and on Day 4 reached a level of pyridoxine uptake that was approximately 85% of controls. Therefore, the mechanism of resistance of Cl.10 cells to high levels of pyridoxine was not due to a permeability mutation of the parental cell line. However, cells that had reverted with respect to pyridoxine permeability still remained resistant to killing.

Growth Rate of Fu5-5 and Cl.10R Cells in 5 mM Pyridoxine Medium. Cl.10 cells that had been placed in pyridoxine-free MEM were subsequently plated in MEM, cultured for an additional 2 months, and then analyzed for their ability to concentrate [³H]pyridoxine. These cells took up approximately the same amount of [³H]pyridoxine as the Fu5-5 cells (data not shown) and were classified as Cl.10 revertants (Cl.10R). If the Cl.10R had reverted back to the parental cell type (Fu5-5), they should again be sensitive to the killing effect of high doses of pyridoxine in the medium. To test this possibility, Fu5-5 and Cl.10R cells (1 x 10⁶ cells/flask) were cultured in MEM with or without 5 mM pyridoxine. Cultures were grown for 72 hr and then harvested and counted. The Fu5-5 cells grown in the absence of 5 mM pyridoxine reached 64 x 10⁶ cells/flask (Table 1). Fu5-5 cells grown in the presence of 5 mM pyridoxine showed essentially no growth; instead, they displayed signs of cell death. In contrast to the Fu5-5 cells, Cl.10R cells proliferated at the same rate in either medium. However, it is interesting to note that the overall growth rate of the Cl.10R cells was reduced compared to that of the parental cell line.
Fu5-5 cells had a population-doubling time of 12 hr compared to a population-doubling time of 20 hr for Cl.10R (data not shown). These results indicate that the cloned pyridoxine-resistant cell line has properties distinct from the parental Fu5-5 cell line. Moreover, once established, Cl.10 cells retain their ability to adapt to high levels of extracellular pyridoxine.

Kinetics of $[3^H]$Pyridoxine Uptake into Fu5-5 and Cl.10 Cells. Saturation kinetics for the uptake of $[3^H]$pyridoxine into Fu5-5 and Cl.10 cells after 30 min of incubation is shown in Chart 4. A Lineweaver-Burke plot of the data generated 2 straight lines intersecting on the $y$-axis. $V_{max}$ values for the Fu5-5 and Cl.10 cells were 100 pmol per mg protein per 30 min. The apparent $K_m$ for the transport system in Fu5-5 cells was 0.71 μM, and for the Cl.10 cells it was 12.5 μM.

Intracellular Levels of PL and PLP. It has been suggested that the intracellular accumulation of PLP can regulate its own formation (9). Since the inability of Cl.10 cells to concentrate $[3^H]$pyridoxine was not due to an inoperative pyridoxal kinase enzyme (data not shown), the possibility existed that PLP could be expressing product inhibition of either pyridoxine oxidase or pyridoxal kinase. When the intracellular levels of PL and PLP were measured, Cl.10 cells contained 69 and 48% more PLP and PL, respectively, than did the Fu5-5 cells (Table 2). These high intracellular levels of PL and PLP could account for part or all of the reduced transport and subsequent trapping of $[3^H]$pyridoxine in Cl.10 cells.

Intracellular Levels of ATP in Fu5-5, CI.10, and CI.10R Cells. When the 3 cell lines were assayed for their intracellular ATP concentrations, it was found that Fu5-5 and CI.10R cells had approximately 215 nmol ATP per mg protein whereas the Cl.10 cells had ATP levels of 295 nmol ATP per mg protein (Chart 5). When the Fu5-5 and CI.10R cells were incubated for 24 hr in MEM supplemented with 5.0 mm pyridoxine and then assayed for ATP, the Fu5-5 cells had ATP levels of 210 nmol/mg protein and the Cl.10R cells had ATP levels of 277 nmol/mg protein. These data indicate that Cl.10 cells had 27% more ATP than did the parental cell line. Cl.10R cells had ATP levels equivalent to Fu5-5 cells when in MEM but in contrast to the parental cell line increased their ATP concentration when exposed to MEM supplemented with 5 mm pyridoxine.

Morphology of Fu5-5, Cl.10, and CI.10R Cells. Initially, we thought that Cl.10 cells might show cell surface changes that could be correlated with vitamin B6 treatment. To investigate this possibility, 4 to 5 cultures each of Fu5-5 and Cl.10 cells were prepared for SEM. We were unable, however, to detect any major observable differences in surface configuration between these 2 cell lines (Figs. 4 and 5).

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**Table 1**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Media</th>
<th>72-hr growth rate (no. of cells/flask x 10⁴)</th>
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<tr>
<td>Fu5-5</td>
<td>MEM</td>
<td>64.0</td>
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<tr>
<td>Fu5-5</td>
<td>MEM + 5 mm pyridoxine</td>
<td>3.28</td>
</tr>
<tr>
<td>Cl.10R</td>
<td>MEM</td>
<td>7.8</td>
</tr>
<tr>
<td>Cl.10R</td>
<td>MEM + 5 mm pyridoxine</td>
<td>9.75</td>
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**Table 2**

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<th>Cell line</th>
<th>PLP (nmol/mg protein)</th>
<th>PL (nmol/mg protein)</th>
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<tr>
<td>Fu5-5</td>
<td>0.326 ± 0.06</td>
<td>0.116 ± 0.02</td>
</tr>
<tr>
<td>Cl.10</td>
<td>0.655 ± 0.04</td>
<td>0.172 ± 0.01</td>
</tr>
</tbody>
</table>

*Mean ± S.D. of 4 individual T-75 flasks.*

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Chart 4. Lineweaver-Burke plot of the uptake of $[3^H]$pyridoxine into Fu5-5 and Cl.10 cells. Cells were incubated with increasing concentrations of $[3^H]$pyridoxine for 30 min at 37°. At the end of the incubation period, cells were washed 3 times in PBS and then dissolved in 1 N NaOH. Results represent the mean of 3 T-75 sq cm flasks.

Chart 5. Measurement of ATP levels in Fu5-5, Cl.10, and CI.10R cells. Protocol for the experiment is described in "Materials and Methods." ATP was measured in a luminometer M 1070 in the presence of the bioluminescence reagent, LUMIT. ATP concentration is expressed as nmol/mg protein and represents the means of 3 T-75 sq cm flasks.

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**Pyridoxine Resistance in Hepatoma Cells**
Our observation that ATP levels were increased in CI.10 cells suggested that pyridoxine might be affecting the mitochondrial compartment. Thus, the fine structure of Fu5-5 and CI.10 cells was examined after preparation for TEM. CI.10 cells appeared to contain a larger volume of mitochondria than did Fu5-5 cells (Figs. 1 and 2). To verify this qualitative observation, the percentage of the cell volume occupied by mitochondria was determined by stereological methods for each of the cell lines. In Fu5-5 cells, 6.31% of the cell volume was occupied by mitochondria; whereas in CI.10 cells, this compartment constituted 9.92% of the fractional cell volume (Table 3). Therefore, the percentage of volume of mitochondria in CI.10 cells was 50% greater than that of the parental cell line. Interestingly, the mitochondrial volume of cells grown in pyridoxine-deficient medium resembled that of the parental cell line Fu5-5 (data not shown).

Biochemical measurements indicated that CI.10R cells cultured in MEM displayed levels of ATP similar to those of Fu5-5 cells. CI.10R cells responded markedly to 5 mM pyridoxine treatment by increasing their ATP levels. In contrast, the levels of ATP in Fu5-5 cells remained essentially constant even when they were exposed to high doses of the vitamin. Again, stereology was used to determine if the mitochondria of CI.10R cells were involved in the observed phenomenon. In CI.10R cells, mitochondria accounted for 10.11% of the fractional cell volume. This value was virtually identical to that for CI.10 cells and represented a 50% increase over that for Fu5-5 cells, the parental cell line.

Analysis of variance indicated that there were significant differences in the fractional cell volume of mitochondria among the 3 cell lines (F = 36.4; d.f. = 2.11; p < 0.001). Although the means for the percentage of the cell occupied by mitochondria in CI.10 and CI.10R cells did not differ from each other, that for Fu5-5 cells was significantly different from both CI.10 and CI.10R (p < 0.01).

On the whole, the mitochondria of CI.10 and CI.10R cells also differed in morphology from those of the parental cell line, although there was some overlap in the appearance of this organelle among all cell lines. Overall, the mitochondria of Fu5-5 cells were smaller than those of either CI.10 or CI.10R cells. Some pyridoxine-treated cells contained mitochondria that were up to 3 to 4 times larger than those of the parental cell line. In Fu5-5 cells, the mitochondrial matrix was generally dense (Fig. 1), while in CI.10 and CI.10R cells it was typically pale and flocculent (Figs. 2 and 3). Cristae were relatively sparse and were usually randomly oriented in mitochondria of Fu5-5 cells. Although cristae were sparse and often randomly arranged in mitochondria of pyridoxine-treated cells, they were also commonly aligned in rows perpendicular to the surface of the mitochondrion (Fig. 2). In addition, the CI.10 and CI.10R cells had an unusual configuration of cristae in which one or more were closely packed and near to or hugging the edge of the mitochondrion (Fig. 3, inset). Mitochondrial granules were not prominent in any of the cell lines. All cultures examined were prepared for microscopy at the same time, making it unlikely that the observed differences in mitochondrial morphology were the result of variations in tissue processing.

It is interesting that the changes seen in mitochondria of cells cultured in the presence of pyridoxine (CI.10) persisted even when these cells were subsequently cultured without this vitamin (CI.10R). This implies that the alterations in mitochondrial structure brought about by pyridoxine treatment were of a permanent nature.

**DISCUSSION**

We have recently shown that vitamin B6 may be a potential antineoplastic agent (5). Data presented in study clearly indicate that cells grown in a medium supplemented with 5 mM pyridoxine display signs of severe growth inhibition and ultimately are killed. However, the precise mechanism by which vitamin B6 retards the growth of cells was not ascertained. In the present communication, studies were undertaken in an attempt to elucidate this mechanism(s).

Our initial attempts to increase the intracellular levels of PLP by culturing cells in high concentrations of pyridoxine resulted in either cell death or retardation of cell growth (Chart 1). It was necessary to adjust culture conditions to diminish toxicity

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Percentage of cell volume occupied by mitochondria in Fu5-5, CI.10, and CI.10R cells</th>
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<tr>
<td>Cell line</td>
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<td>Fu5-5</td>
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<td>3</td>
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<td>4</td>
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</table>

* Each number designates an individual culture.

* The fractional cell volume for mitochondria was determined by stereological methods as described in "Materials and Methods." The maximum number of test points for 16 cells is 1728. However, the actual number may be smaller since points falling over extracellular space are not tabulated.

* Average ± S.D. of values in Column 5. The mean for Fu5-5 is significantly different from those for both CI.10 and CI.10R (p < 0.01).

* The means for CI.10 and CI.10R do not differ significantly.
to the cells. This was accomplished by increasing the concentrations of pyridoxine in the medium over a period of 3 to 4 months. Using this protocol, a cell line was established that was resistant to killing by 5 mM pyridoxine. The experiments presented in this communication describe this cell line (CI.10) and suggest its value for the study of the regulation of cell growth by vitamin B₆.

The CI.10 cell line may prove a valuable tool in other areas of research, such as studies of vitamin B₆ metabolism. It has been suggested by Mehansho et al. (13) that pyridoxine is passively transported into the cell and trapped by phosphorylation to pyridoxine 5'-phosphate, a reaction catalyzed by pyridoxal kinase. Kwok and Churchich (9) have shown that both pyridoxal kinase and pyridoxine oxidase are involved in the regulation of PLP, and Li et al. (10) have shown, in vitro, that the control mechanisms regulating the cellular content of PLP operate even when vitamin B₆ is present in excess. We have shown here that it is possible to increase the intracellular concentration of PLP by culturing cells in an excess of vitamin B₆. Kinetic studies revealed that Vₘₐₓ for the uptake of [³H]pyridoxine in CI.10 cells was the same as in the parental line (Chart 4). However, the apparent Kₘ for the transport system in CI.10 cells was 12.5 μM, almost 18 times higher than for Fu5-5 cells. Since both cell lines had approximately the same level of pyridoxal kinase activity (data not shown), it is possible that the reduced uptake of [³H]pyridoxine into CI.10 cells was the result of product inhibition by either PLP or PL or both on the enzymes involved in the intracellular trapping of pyridoxine.

However, we realize that other factors may be contributing to the reduced cellular uptake of [³H]pyridoxine. Since we have not investigated the intracellular metabolism of PLP, it may be possible that the enzymes responsible for the synthesis and/or degradation of PLP have been altered or inhibited to some degree resulting in an accumulation of PLP metabolites. In the present study, only intracellular PL and PLP were measured. Therefore, it is possible that other vitamin B₆ metabolites (pyridoxamine, pyridoxamine phosphate, pyridoxine phosphate) could play a role in the regulation of the cellular uptake of [³H]pyridoxine. Another factor which merits consideration is the possibility that the total intracellular protein concentration could be reduced in CI.10 cells thus affecting not only the amount of PLP bound to protein but also the osmotic activity of the cell. Therefore, it is clear that more intensive investigation must be carried out on CI.10 cells before definitive conclusions can be drawn concerning vitamin B₆ uptake and metabolism in this cell line. Nevertheless, it is important to bear in mind that the defective pyridoxine uptake developed in CI.10 cells can be reverted to a level approaching that of the parental cell by removal of pyridoxine from the medium, all this while the apparent mutation of resistance to killing is retained.

Another point that merits discussion is the possible role of PLP in the regulation of glycolysis. Srivastava and Beutler (18) have shown that, in RBC, PLP inhibits hexokinase, phosphofructokinase, and pyruvate kinase, 3 enzymes involved in glycolysis. PLP has also been shown to inhibit lactate production in rat liver supernatant fluid, possibly through the inhibition of hexokinase (7).

The relevance of PLP inhibition of hexokinase to the present work can be best appreciated when related to other available information. Hepatoma cells characteristically have abnormally high hexokinase activity (2) which is associated with the mitochondrial fraction. Coupled to the elevated hexokinase activity are alterations in the glycolytic rate and changes in mitochondrial volume (3).

Two of our observations suggest the possible inhibition of hexokinase by the increased levels of PLP in CI.10 cells. (a) Pyridoxine treatment leads to a marked decrease in the growth rate of CI.10 cells. The population-doubling time of CI.10 cells was 24 hr, while that of Fu5-5 cells was 12 hr. The slower growth rate of CI.10 cells could result if PLP was inhibiting glycolysis by blocking the activity of the glycolytic enzymes mentioned above. (b) We found that CI.10 cells had both increased levels of ATP and a 50% increase in the mitochondrial volume compared to the parental cell line. These findings lend further support to our theory that PLP may inhibit the activity of hexokinase, with the consequent slowing of the glycolytic rate. Although conclusive evidence is lacking, additional support for our hypothesis comes from the work of Jayaraman et al. (8). In their studies, high levels of glucose suppressed the formation of mitochondria, whereas when the concentration of this sugar was reduced functional mitochondria were formed.

Although we have not described the exact molecular mechanism of pyridoxine resistance, the newly established cell line (CI.10) has 69% more intracellular PLP, 27% more intracellular ATP, and a 50% increase in the volume of the cell occupied by mitochondria as compared to the parental cell line (Fu5-5). These parameters ultimately may be involved in the mechanism of resistance. Also, it is conceivable that pyridoxine resistance may be traceable to a genetic alteration, providing an additional marker in somatic cell genetics.

REFERENCES

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Fig. 1. TEM of Fu5-5 cell cultured in MEM. Note that the mitochondria (m) of Fu5-5 cells are modest in size, have a dense matrix, and contain relatively sparse, randomly oriented cristae. er, rough endoplasmic reticulum; n, nucleus. Uranyl acetate and lead citrate, × 15,000.

Fig. 2. TEM of Cl.10 cells grown in MEM containing 5 mM pyridoxine. Note that, in contrast to Fu5-5 cells, the mitochondria (m) of Cl.10 cells are large and contain a pale, flocculent matrix. er, rough endoplasmic reticulum; n, nucleus. × 15,000. Inset, many mitochondria of pyridoxine-treated cells containing cristae (arrowheads) that are aligned in parallel rows. Uranyl acetate and lead citrate, × 27,800.
Fig. 3. TEM of Cl.10R cell cultured in MEM. Note that, even in the absence of high concentrations (mM) of pyridoxine in the medium, mitochondria (m) of Cl.10R cells do not revert to the morphology typical of the parental cell line, Fu5-5. Instead, they resemble the mitochondria of Cl.10 cells; i.e., they are large with a pale, floucculent matrix. er, rough endoplasmic reticulum; n, nucleus. × 15,700. Cristae in mitochondria of both Cl.10 and Cl.10R cells may have an unusual arrangement; one (arrowheads) or more cristae hug the edge of the mitochondrion (inset). Uranyl acetate and lead citrate, × 27,800.

Fig. 4. Scanning electron micrographs of Fu5-5 cells, showing their typical surface morphology. The surfaces of Fu5-5 cells are studded with many microvilli which vary in length. × 1800.

Fig. 5. Scanning electron micrographs of Cl.10 cells, showing their typical surface morphology. Note that the surfaces of Cl.10 cells do not differ markedly from those of the parental cell line Fu5-5, although the microvilli of Cl.10 cells tend to be short and of relatively uniform length. × 1800.
Pyridoxine Resistance in a Rat Hepatoma Cell Line

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