Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 3304–3308, April 1993
Medical Sciences

Inhibition of rate of tumor growth by creatine and cyclocreatine

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Contributed by Mildred Cohn, December 28, 1992

ABSTRACT **Growth rate inhibition of subcutaneously implanted tumors results from feeding rats and athymic nude mice diets containing 1% cyclocreatine or 1%, 2%, 5%, or 10% creatine. The tumors studied included rat mammary tumors (Ac33tc in Lewis female rats and 13762A in Fischer 344 female rats), rat sarcoma MCI in Lewis male rats, and tumors resulting from the injection of two human neuroblastoma cell lines, IMR-5 and CHP-134, in athymic nude mice. Inhibition was observed regardless of the time experimental diets were administered, either at the time of tumor implantation or after the appearance of palpable tumors. For mammary tumor Ac33tc, the growth inhibition during 24 days after the implantation was ≈50% for both 1% cyclocreatine and 1% creatine, and inhibition increased as creatine was increased from 2% to 10% of the diet. For the other rat mammary tumor (13762A), there was ≈35% inhibition by both 1% cyclocreatine and 2% creatine. In the case of the MCI sarcoma, the inhibitory effect appeared more pronounced at earlier periods of growth, ranging from 26% to 41% for 1% cyclocreatine and from 30% to 53% for 1% creatine; there was no significant difference in growth rate between the tumors in the rats fed 1% and 5% creatine. The growth rate of tumors in athymic nude mice, produced by implantation of the human neuroblastoma IMR-5 cell line, appeared somewhat more effectively inhibited by 1% cyclocreatine than by 1% creatine, and 5% creatine feeding was most effective. For the CHP-134 cell line, 33% inhibition was observed for the 1% cyclocreatine diet and 71% for the 5% creatine diet. In several experiments, a delay in appearance of tumors was observed in animals on the experimental diets. In occasional experiments, neither additive inhibited tumor growth rate for the rat tumors or the athymic mouse tumors.**

The brain isozyme of creatine kinase (CK-BB) has been shown to be associated with growth phenomena in a number of systems. Estrogen injection into immature rats causes a doubling of the weight of their uteri in 24 hr and, within 1 hr after estrogen injection, the concentration of mRNA for uterine CK-BB begins to increase (1). Other hormones and growth factors stimulate CK-BB activity in their target cells (2). Increased CK activity has been observed in regenerating rat liver (3), and CK has been implicated in spindle activity during normal cell division (4). In many tumors, although not all, CK-BB activity is considerably higher than in the tissue of origin (3, 5); its elevated activity has been used as a diagnostic marker and prognostic indicator for small-cell lung carcinoma (6) and neuroblastoma (7). Recently it has been reported that CK-BB may be induced by the oncogenic products of the E1a region of adenovirus type 5 (8), and the intriguing possibility that induction of CK-BB may be related to metabolic events following oncogenic activation was suggested.

One approach for probing the metabolic role of CK in tumor tissue is to replace creatine in the tissues with an analog of creatine that inhibits the synthesis of creatine and exhibits markedly different kinetic and thermodynamic properties from creatine. Walker (9) has extensively investigated the effects of just such a creatine analog, cyclocreatine (1-carboxymethyl-2-iminoimidazolidine), on various organs in intact animals and in Ehrlich ascites cells. Cyclocreatine was first synthesized by Kenyon and coworkers (10), and it was shown to be an excellent substrate for rabbit muscle CK (11). Annesley and Walker (12) found surprisingly that the ratio of equilibrium constants for the CK reaction with cyclocreatine as substrate relative to creatine was 26 in favor of the phosphagen, and the result was confirmed by a ^{31}P NMR determination (13). By the V_{max}/K_m criterion of substrate efficiency, creatine is ≈6-fold better than cyclocreatine with rabbit skeletal muscle CK (12), but, as might be expected from the relative equilibrium constants, V_{max}/K_m for phosphocreatine is 160-fold greater than that of phosphocyclocreatine.

Our interest in investigating the effect of cyclocreatine on tumor growth stemmed from the observation that it is toxic to chicken embryos (14) and to developing rat fetuses (9, 15) in the early stages of their development. Creatine was included in the studies as a control for the effect with cyclocreatine. In this report, the inhibitory effects on tumor growth rate resulting from feeding 1% cyclocreatine or 1%, 2%, 5%, or 10% creatine in the diet to rats with implanted subcutaneous tumors (two mammary tumors and one sarcoma) and to athymic nude mice implanted subcutaneously with human neuroblastoma cells are presented.

MATERIALS AND METHODS

Animals and Tumors. Tumors in inbred rats used in these experiments include 13762A in Fischer 344 female rats, Ac33tc in Lewis female rats, and MCI in Lewis male rats. The tumors designated 13762A (16) and Ac33tc are both metastasizing mammary tumors. Ac33tc was derived from the tissue culture of a tumor that arose after the treatment of rats with the anticancer compound dimethyl-β-aziridinoproprionamide (17, 18). The original tumor was not metastatic, whereas the one derived from its tissue culture metastasizes to the regional lymph nodes and lungs. MCI (19) is a sarcoma induced in Lewis rats by injection of methylcholanthrene. The Fischer 344 rats were purchased from the National Cancer Institute, Frederick Cancer Research and Development Center (Frederick, MD). Lewis rats for carrying tumors were bred in a closed animal colony, and additional rats were purchased when needed from Harlan–Sprague–Dawley. All rats used weighed 100–150 g.

Six-week-old weanling athymic NCR-NU mice were also obtained from the National Cancer Institute (Frederick, MD). The neuroblastoma cell lines used were obtained from the laboratory of Roger Kennett (University of Pennsylvania). Two lines were investigated, IMR-5 (20), a subclone of IMR-32 (21) from the Institute of Medical Research (Camden,

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Abbreviations: CK, creatine kinase; CK-BB, brain isozyme of CK.
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NJ), and CHP-134, derived in the research laboratories of the Children's Hospital of Philadelphia by Harvey Schlesinger.

Implantation of Tumors. To obtain 13762A cells for subcutaneous transplants, 13762A ascites tumor was grown by injecting 1–2 × 10^7 cells in RPMI medium interperitoneally in female Fischer 344 rats, and the ascites cells were harvested after 1 week. The ascites tumor 13762A was adapted to this growth form from the solid tumor 13762NF (16) by the A. E. Bogden laboratory of the Mason Research Institute (Worcester, MA). Lewis rat tumors were maintained by trocar transplants as solid tumors, and single-cell suspensions were prepared in Hanks' balanced salt solution by forcing minced tumor tissue fragments through a 40-mesh stainless steel sieve. The implantation of all rat tumors was made subcutaneously in a volume of 0.5 ml, with 0.5 × 10^6 live cells, based on trypan blue dye exclusion. All transplants were made using sterile technique.

The neuroblastoma cells were cultured in 150-ml flasks with modified Dulbecco's medium containing 10% (vol/vol) fetal calf serum. They were maintained in a humidified atmosphere of 92% air/8% CO_2. At 90% confluence, the cells were harvested with the addition of the sodium salt of EDTA. For the athymic mice, 0.6–0.8 × 10^7 cells were injected subcutaneously on each side of the lower back.

Experimental Diets. Cyclocreatine was synthesized as described by Wang (22), and creatine was purchased from Sigma. The rats were fed Purina Lab Chow 5001 (Buckshire Feeds, Lansdale, PA) ad libitum with no addition or admixed with cyclocreatine (1%, wt/wt) or creatine (1%, 2%, 5%, or 10%, wt/wt). Unless otherwise specified, the food in the form of chow meal was fed to the rats from standard glass food dishes. In some experiments, the compounds were incorporated with the same chow meal into pellets by Dyets (Bethlehem, PA). After eating Purina Lab Chow pellets for 2 or 3 weeks after arrival in the lab facility, the mice were fed ground Purina Mouse Chow 5015 with no addition or admixed with 1% cyclocreatine or 5% creatine. They were housed in filter-top cages.

Evaluation of Tumor Growth. Measurements of tumor size were made in three dimensions, $d_1 \times d_2 \times d_3$, with centimeter calipers (23) at 3- to 4-day intervals. The hair was shaved from the tumor area to facilitate measurements. Calculations of tumor volume were made by multiplying the product of the three dimensions by $\pi/6$. Standard deviations were used to obtain the SEM of each experimental group.

RESULTS

In Fig. 1, the average volumes of the subcutaneously implanted rat mammary adenocarcinoma Ac33tc tumors of three groups of animals with three animals in each group—the control group, the group with 1% creatine, and the group with 1% cyclocreatine added to the diet—are shown as a function of time over a period of 24 days after implantation. Tumors developed 3–6 days after implantation in all groups. Cyclocreatine depressed the growth over the time course by ≈50% (range 46–54%), and creatine also depressed the growth ≈50% (range 37–65%). Repeated experiments with this tumor gave similar results.

A dose–response experiment with littermates was done only with creatine at 2%, 5%, and 10%; there were two rats in the groups fed 2% and 5% creatine and only one rat fed 10% creatine. Cyclocreatine above 1% is reported to be toxic (24), but creatine is apparently not toxic to humans even at 15 g per day (25). The increasing inhibition of the growth rate of the subcutaneously implanted Ac33tc tumor with increasing creatine levels in the diet is shown in Fig. 2; at 22 days after implantation, for example, the inhibition of growth by 10% creatine in the diet was almost 90%. It should be noted that the urine of animals fed 10% creatine in the diet appeared

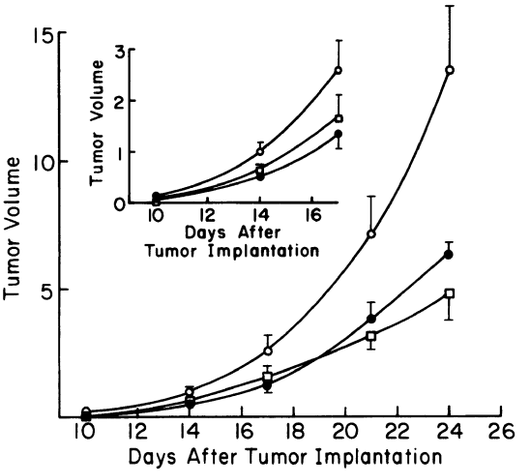

FIG. 1. Tumor growth curves for mammary adenocarcinoma Ac33tc for 24 days after implantation in female Lewis rats. There were three rats in each group. ○, control; ●, 1% cyclocreatine; □, 1% creatine. The error bars correspond to SEMs. (*Inset*) Expansion of the ordinate for days 10–16 after tumor implantation.

milky, probably due to the excretion of excess insoluble creatine; those animals fed 5% creatine in the diet had a smaller amount of insoluble material suspended in the urine. The results on inhibition were striking as shown in the photograph in Fig. 3, even though only a small number of animals were involved in this experiment as indicated in Fig. 2.

The effects of cyclocreatine and of creatine were also investigated for the mammary tumor 13762A with six Fischer 344 female littermates in each group. There was a slight delay in tumor development on the experimental diets. The first two tumors appeared in the control group on day 7 after

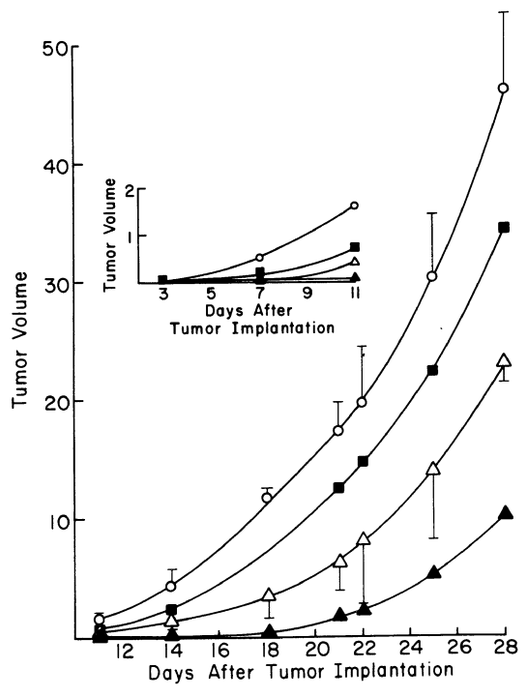

FIG. 2. Dose–response growth curves for tumor Ac33tc for 28 days after implantation in Lewis female littermates. There were two rats in the groups fed 2% and 5% creatine and one rat fed 10% creatine. ○, control; ■, 2% creatine; △, 5% creatine; ▲, 10% creatine. One of the rats fed 2% creatine died accidentally on day 15. (*Inset*) Expansion of the scales for days 3–11 after tumor implantation.

FIG. 3. Photograph of rats on day 15 in the experiment shown in Fig. 2. The rats shown (from left to right) are from the control, 2% creatine, 5% creatine, and 10% creatine groups.

implantation, and all six had developed on day 12; for the 1% cyclocreatine group, one tumor was palpable on day 6, and all six had developed on day 14; for the 2% creatine group, one tumor was observed on day 7, and all six had developed on day 14. As can be seen in Fig. 4, 1% cyclocreatine and 2% creatine had similar inhibitory effects on growth rate within experimental error; at 25 days, 1% cyclocreatine inhibited ≈35% and 2% creatine inhibited 30%, but at 20 days 1% cyclocreatine inhibited 33% and 2% creatine inhibited 43%.

Another type of tumor, the MCI sarcoma, was implanted in Lewis male littermates, six in each group, which were fed experimental diets in the form of pellets containing 1% cyclocreatine or 1% or 5% creatine. All animals had developed tumors at day 10 after implantation. Within experimental error, 1% cyclocreatine and 1% creatine were equally effective; the data for the 5% creatine diet did not differ significantly from the 1% creatine diet, unlike the results with the Ac33tc mammary tumor. There was a trend for the creatine to be more effective at earlier times; for example, at 16 days after implantation, the 1% creatine diet yielded ≈53% inhibition and the 1% cyclocreatine resulted in 41% inhibition, but at 20 days after implantation, inhibition was ≈30% for creatine and 26% for cyclocreatine.

With the MCI sarcoma, the effect of feeding 5% creatine after the tumor appeared rather than at the time of implantation was examined. The tumor growth curves in Fig. 5 show the average volumes of six tumors in the control group and of eight tumors in animals fed 5% creatine beginning the sixth day after implantation, when the tumors first appeared, and continuing until 20 days after implantation. The creatine diet caused ≈40% inhibition.

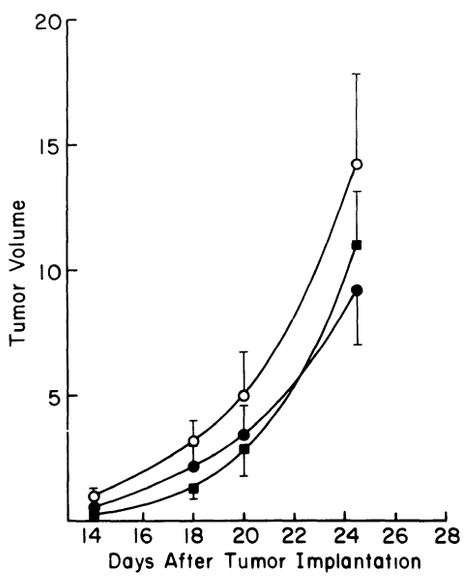

FIG. 4. Tumor growth curves for mammary adenocarcinoma 13762A for 25 days after implantation in Fischer 344 female littermates (six in each group). ○, control; ●, 1% cyclocreatine; ■, 2% creatine.

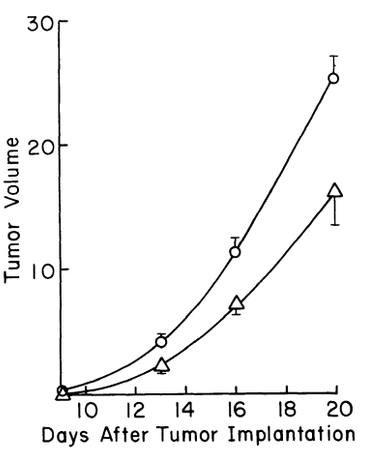

FIG. 5. Tumor growth curves for rat sarcoma MCI in Lewis male littermates for 20 days after implantation. They were fed the 5% creatine diet, which was initiated 6 days after implantation, when tumors become palpable. There were six rats in the control group and eight rats in the 5% creatine group. ○, control; △, 5% creatine.

The efficacy of dietary cyclocreatine and creatine as inhibitors of human neuroblastoma tumor growth rate was tested in athymic nude mice. Three groups with three or four animals in each group were implanted with two injections of CHP-134 cells, one on each side of the lower back in each animal. The effect of 5% creatine and 1% cyclocreatine in diets, initiated at the time of implantation, was most strikingly reflected in the delay in the development of tumors. Of the eight injections in each group of four animals in the control and cyclocreatine groups and of the six injections in three animals in the creatine group, on day 19 after implantation, the control group had formed tumors in four of eight, the cyclocreatine group in two of eight, and the creatine group in two of six. At day 38, six of eight in the control group, three of eight in the cyclocreatine group, and three of six in the creatine group had developed tumors. Eventually all developed tumors.

The usual experimental design was modified in a second experiment with the CHP-134 neuroblastoma cell line. To ensure that a sufficient number of tumors would be available in each of the three groups for statistically significant analysis, the mice were not fed the experimental diets until tumors had appeared. Thus, each group (control, 1% cyclocreatine-fed, and 5% creatine-fed) consisted of eight tumors. The tumor volume of each individual tumor was plotted as a function of the number of days on the diet. The values of the tumor volumes of each group plotted in Fig. 6 are averages of the sizes of individual tumors in each group, taken from the volume–time curves of the individual tumors on the days indicated. As shown in Fig. 6, the average growth rate over 26 days of feeding was inhibited 33% (range 21%–45%) by cyclocreatine and 71% (range 66%–78%) by creatine.

A similar experiment was done with the neuroblastoma cell line IMR-5 with four groups of athymic mice: control, 1% cyclocreatine-fed, 1% creatine-fed, and 5% creatine-fed. There were four animals in each group (each animal had two

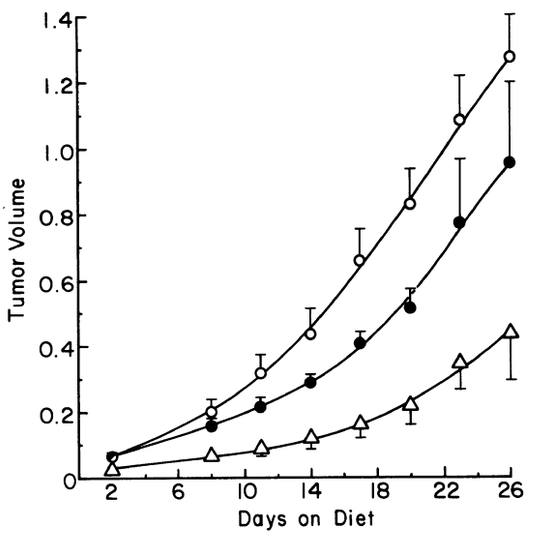

FIG. 6. Tumor growth curves (volume versus days on diet) for athymic mice implanted with human neuroblastoma cells (CHP-134). Experimental diets were initiated when tumors became palpable. Growth curves were plotted for individual tumors, and tumor volumes were taken at 3-day intervals (2–26 days) from the individual curves and averaged for each group to obtain the tumor volume shown in the figure for control (○), creatine-fed (△), and cyclocreatine-fed (●) mice, respectively. The number of tumors averaged were as follows: control group, eight for days 2–20 and seven for days 23 and 26; cyclocreatine group, eight for days 2–14, six for days 17–20, and four for days 23 and 26; creatine group, eight for days 2–20, seven for day 23, and six for day 26.

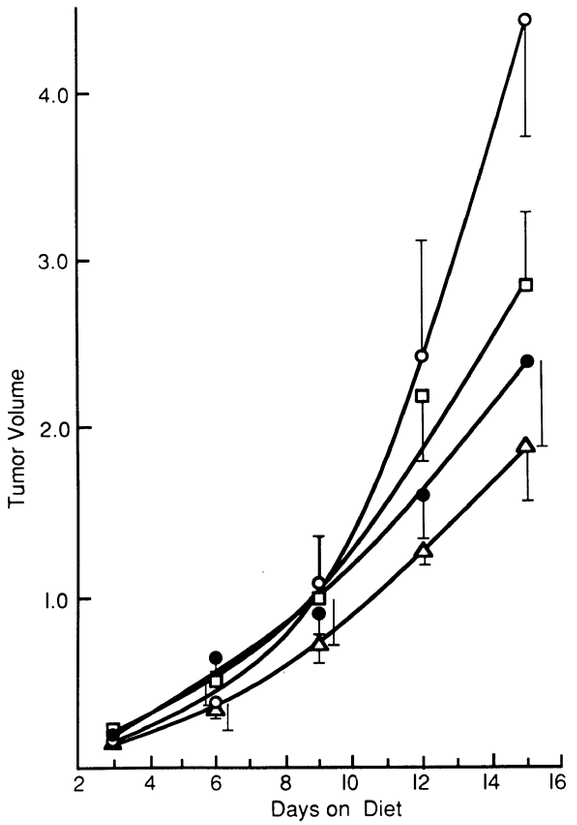

FIG. 7. Tumor growth curves for athymic mice for 15 days after implantation of IMR-5 neuroblastoma cells. The number of tumors averaged were as follows: control group, seven for all days; cyclocreatine group, eight for day 3, six for days 6 and 9, and five for day 12; 1% creatine group, eight for all days; 5% creatine group, six for days 3–12, and four for day 15. ○, control; ●, 1% cyclocreatine; □, 1% creatine; △, 5% creatine. Several animals in the cyclocreatine and 5% creatine groups died accidentally on day 32.

tumors), and the experimental diets were initiated when the tumors appeared. The values of tumor volume plotted in Fig. 7 are averages obtained from volume–time curves of individual mice as described for the CHP-134 experiment above. Inhibition of the tumor growth rate for the IMR-5 tumor increased with time: from day 9 to day 12, the percent inhibition ranged from 10% to 36% for the 1% creatine group, from 16% to 46% for the 1% cyclocreatine group, and from 33% to 57% for the 5% creatine group. At the same concentration in the diet (1%), cyclocreatine was more effective than creatine, but 5% creatine in the diet was most effective.

All the rats gained weight during the course of the experiments, but in most cases, the rats fed cyclocreatine gained less weight than the control or creatine-fed rats. With all the tumor types included in this study, the three rat tumors and the two neuroblastoma tumors, there were occasional experiments that were negative (i.e., neither cyclocreatine nor creatine inhibited growth). We have no explanation for these failures.

DISCUSSION

It may be concluded from the experiments presented that creatine and its analog cyclocreatine are both usually effective in inhibiting the growth rate of a variety of tumors as exemplified in this study by two rat mammary tumors, one rat sarcoma, and two human neuroblastoma tumors in athymic nude mice. Quantitatively the effects differ; in the Ac33tc mammary tumor the inhibition increased as creatine concen-

tration increased from 2% to 10%, but in the MCI rat sarcoma, there was no significant difference in growth rate inhibition between the tumors of animals fed 1% and 5% creatine in the diet, respectively. The human neuroblastoma tumor (IMR-5) was dose-dependent for creatine, and at 1% in the diet, cyclocreatine was more effective than creatine. For both neuroblastomas, 5% creatine was more effective than 1% creatine.

The inhibition of tumor growth rate by cyclocreatine or by creatine was observed when feeding commenced, 2 weeks before implantation (data not shown), at the time of implantation, or at the time tumors appeared. This suggests that accumulation in the tissue is not essential for the inhibitory effect. Walker (9) reported that, with 1% cyclocreatine in the diet of rats, several weeks of feeding are required to replace the creatine with cyclocreatine. The difference in the cyclocreatine thermodynamics in the CK reaction is probably irrelevant to its inhibitory property described here. If creatine and cyclocreatine share the same mechanism, obviously the difference in the thermodynamics and kinetic properties in the CK reaction cannot be correlated with their similar inhibitory effects. However, the two compounds may not operate by the same mechanism, since preliminary experiments with cultured cell lines (not shown) reveal cytotoxicity when cyclocreatine but not creatine is added to the medium.

It is not clear whether the phosphorylated or the nonphosphorylated form of creatine and its analog are the active components producing the growth inhibition. One known effect of the two nonphosphorylated forms is the inhibition of the transamidination step in the biosynthesis of creatine, which would result in sparing arginine, the limiting precursor. There is a possibility that the increased level of arginine could lead to the increased formation of nitric oxide (26), which is one of the factors activating macrophages (27).

Preliminary experiments using ^{31}P NMR spectroscopy of the CHP-134 neuroblastoma tumor in athymic mice reveal dramatically higher phosphagen to inorganic phosphate and phosphagen to ATP ratios in the cyclocreatine-fed animals than in the controls, indicating paradoxically a more satisfactory bioenergetic state in the growth-inhibited tumors. The value of phosphocreatine to inorganic phosphate in the tumors of the creatine-fed animals falls between the other two groups, but closer to the control groups. If phosphocyclocreatine or phosphocreatine are the active forms, they may be slowing tumor growth by inhibition of glycolysis. It has been established that phosphocreatine inhibits enzymes in the glycolytic pathway including glyceraldehyde-3-phosphate dehydrogenase (28), phosphofructokinase (29, 30), and pyruvate kinase (31). It is not known whether phosphocyclocreatine also inhibits these enzymes. It has been suggested by Kemp (31) that the characteristics of muscle pyruvate kinase are most suitable for regulating glycolytic flux in muscle.

The ratio of the phosphomonoester peak to that of ATP in ^{31}P NMR of the neuroblastoma is much lower in the cyclocreatine-fed animals than in the controls, and again the ratio for the creatine-fed animals is intermediate between the other two groups. ^{31}P NMR studies of neuroblastoma in patients have shown that both the phosphomonoester peak, largely phosphoethanolamine, and the phosphomonoester to ATP ratio fall with tumor regression, spontaneous or treated, and increase in relapse (32). Further studies with ^{31}P NMR and ^{13}C NMR should aid in elucidating the metabolic role of the phosphagens in tumors. Many questions remain including, is the inhibition of tumor growth rate due to a direct metabolic role of the phosphagens or an indirect role in other cellular processes? Is the phosphorylated form the active one, and is the role of the level of CK solely to insure the rapid phosphorylation of creatine or cyclocreatine?

This work was supported by a grant from the James S. McDonnell Foundation to J. S. Leigh (Department of Biochemistry and Biophysics, University of Pennsylvania) and the Children's Cancer Research Foundation of Philadelphia.

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