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Alterations in Serum Levels, Anti-tumor Activity and Toxicity of Methotrexate in Rats After a Short Period of Nutritional Depletion

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Abstract—*The alterations in serum levels, anti-tumor activity and host toxicity of methotrexate (MTX) were tested in tumor bearing rats following a period of dietary manipulation. A protein deprived (PD) diet or a diet containing a normal protein content (NP) was administered for 5 days and MTX injected intra-peritoneally (i.p.) at the end of the 5 day period.*

The MTX serum levels were significantly elevated in rats which received the PD diet, as compared to NP dietary rats. This elevation correlated with an enhanced tumor response to MTX administration. In addition, bone marrow toxicity and intestinal tract toxicity, measured with flow cytometry (FCM) of the bone marrow and morphometry of the jejunal mucosa respectively was increased in rats receiving the PD diet.

These results indicate that the serum clearance of MTX is delayed in animals suffering from malnutrition, leading to both enhanced tumor response and increased host toxicity.

INTRODUCTION

THE TOXIC side-effects of chemotherapy can be so severe that the effectiveness of treatment will be hampered by the necessity to limit both dosage and duration of therapy. Especially in malnourished cancer patients a potentially useful regimen frequently has to be terminated prematurely because of serious toxic side-effects.

Malnutrition in cancer patients is common and has been identified as an important and independent factor prognostic of survival in a variety of cancers [1–5]. In spite of a vast literature on the subject, the mechanism by which tumors induce malnutrition remains unknown. Most theories indicate alterations in host energy and/or nitrogen metabolism, with or without concomitantly reduced food intake [6, 7].

It has been suggested that in malnourished animals the plasma clearance of chemotherapeutic drugs is delayed in comparison to well-fed controls. This observation might offer an explanation for the increased toxicity of chemotherapeutic drugs that malnourished cancer patients experience [8–11].

The purpose of the present study was to evaluate the potential alterations in methotrexate (MTX) serum levels, host toxicity and tumor response when MTX was administered after a short period of nutritional depletion. Nutritional depletion was achieved by feeding these rats a protein deprived (PD) diet, while rats consuming a diet with a normal protein (NP) content served as controls.

MATERIALS AND METHODS

Young female WAG/Rij rats were bred in the animal facilities of the Netherlands Cancer Institute under specific pathogen free (SPF) conditions and used at an initial weight of 150–160 g. All rats were maintained in a temperature controlled room on a 12 h light–12 h dark schedule. They were housed two rats to a cage and were fed one of the two study diets and water *ad libitum*.

The effects of the dietary protein content were assessed at two levels of protein in the diet: no protein (PD diet) and normal (27%) protein content (NP diet, both diets from Hope Farms, Woerden, The Netherlands) (Table 1). The PD diet contained 75.5% dextrose, the NP diet 47.3%. Both diets contained identical amounts of additives and were

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Table 1. Composition of the two diets used for nutritional manipulation. PD: protein-deprived diet, NP: diet with a regular protein content. Diets are iso-caloric and contain identical amount of additives

	PD	NP
Mixed protein*	0.0	27.0
Dextrose (glucose)	75.5	47.3
Corn starch	10.0	10.0
Alpha-cellulose	5.0	5.0
KH_2PO_4	0.8	0.8
KCl	0.4	0.4
$CaCO_3$	1.2	1.2
$CaHPO_4.2H_2O$	0.7	0.7
MgO	0.2	0.2
Choline chloride 50%	0.3	0.3
Vitamins and trace element mix	1.0	1.0
NaCl iodized	0.4	0.4
Soya/sunflower oil 50/50	4.5	4.5
Lard	0.0	1.2
	100%	100%

*Mixed protein — 79% crude protein

Casein	26
Lactalbumin	10
Wheat gluten	14
Corn gluten	5
Meat meal	30
Meat protein solubles	5
Fish meal	5
Soya protein 62%	5
	100%

Diets from Hope Farms, Woerden, The Netherlands.

iso-caloric. Food intake was calculated daily by weighing the distributed and remaining food pellets. The design of the experiment is shown in Fig. 1. On day 0 the rats were inoculated subcutaneously in the flank with 1×10^6 R1 rhabdomyosarcoma cells in a single cell suspension. R1 is a non-metastasizing tumor with constant and well-defined growth characteristics *in vivo* and *in vitro* [12, 13]. All rats were initially fed the NP diet for a 3 week period to allow proper tumor development. On day 21, 54 rats with tumors suitable for tumor volume determination were randomized to receive the PD diet ($n=27$) or to continue with the NP diet ($n=27$). On day 26, 50 rats were injected intraperitoneally (i.p. push-injection) with methotrexate (MTX), 30 mg/kg body wt, 5 mg/ml dissolved in sterile water.

Two rats from each dietary group served as controls and were sacrificed at 0 h without receiving MTX. Two rats of each group were killed 2 and 6 h, four rats of each group 24 and 48 h, and three rats 72, 96 and 168 h after MTX administration. From each dietary group four rats were kept alive until day 47 for tumor growth curve construction. Before sacrifice blood was obtained from each rat via cardiac puncture under light ether anesthesia to determine the hemoglobin level (g/100 ml), leucocyte ($\times 10^3/\mu l$) and platelet ($\times 10^9/mm^3$) numbers and serum folinic acid ($\mu g/l$) level. Subsequently the rats were killed by cervical dislocation. Tumors were excized, tumor weight and carcass weight were determined and material for cell kinetic studies was taken from predetermined sites.

Identical parts of each tumor, representing 25% of the tumor volume and containing equal parts of peripheral and central areas, were excized for flow cytometric (FCM) analysis. Tumor samples were mechanically and enzymatically processed to single-cell suspensions, as has been described before [14].

Bone marrow was harvested for FCM studies from the two femora by flushing the bones with phosphate buffered saline (PBS). Cells from femora and tumors were fixed in 70% ethanol. DNA of tumor and bone marrow cells was stained with ethidium bromide and Hoechst stain 33258. FCM histograms of DNA/cell were determined using the ICP 11 flow cytophotometer (Phywe AG, Göttingen, F.R.G.) and used to calculate the percentage of cells in the S-phase of the cell cycle (S-phase cells) by planimetry. The results of the bone marrow FCM measurements are given as the mean of two determinations.

To study small intestinal morphometry standardized jejunal segments were taken from a point just distal to the ligament of Treitz. For light microscopy studies, specimens were fixed in Bouins' solution, sectioned and stained with hematoxylin and eosin.

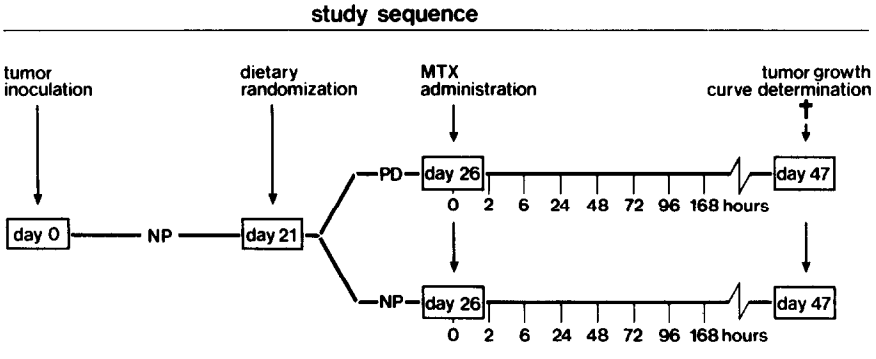

Fig. 1. Experimental design of the study.

Quantitative measurements of villus height and crypt depth (μm) were performed using a Glarex (Zeiss) projection microscope at a 100× magnification and a commercially available graphic tablet (Kontron, Munich, F.R.G.) [15].

Mean total serum methotrexate (MTX) levels (free and protein-bound in $n=16$ rats) (nmol/l) were determined at 0, 2, 6, 24 and 48 h after MTX administration by high pressure liquid chromatography (HPLC) according to the method described by Cairnes and Evans [16]. This method was slightly modified by using a Chrompack Microsphcr C 18 column, particle size 3 μm, 100 × 4.6 mm.

Tumor growth delay curves were determined in eight rats that were not sacrificed until day 47. The tumors were measured in three dimensions three times a week with a Vernier callipers, and tumor volume was determined via the formula $V=\pi/6 \times$ length × width × height. Tumor growth delay was defined as the number of days from the day of MTX administration to the day the tumor had regrown to its original volume.

The results were analyzed statistically using a one-way analysis of variance with and without logarithmic transformation of the data.

RESULTS

Clinical toxicity

All animals showed signs of ill health after MTX administration (dry noses, ruffled fur). No diarrhea was noted. Food intake decreased in both groups during 3–4 days, with the mean food intake in the PD dietary group being 70% of the NP group. The NP group generally showed a quicker recovery and started to regain weight from the 4th day after MTX administration, to reach the pre-MTX weight level after 12 days.

Flow cytometric study (FCM) of bone marrow

The effect of dietary manipulation and MTX administration on the percentage S-phase cells in the bone marrow is depicted in Fig. 2. A significantly decreased percentage of S-phase cells in the bone marrow was seen after dietary manipulation as such (16.9 ± 0.1 in the PD group vs. 29.2 ± 0.9 in the NP group, $P<0.05$). Prolongation of the period of protein deprivation did not lead to a continuing decrease in the percentage S-phase cells (data not shown).

The effect of MTX administration on the bone marrow became clearly evident in both groups after 48 h. In the PD group the percentage of S-phase cells decreased from 16.9 ± 0.1 to 7.4 ± 0.6 ($P<0.01$) and in the NP group from 29.2 ± 0.9 to 9.3 ± 1.3 ($P<0.001$). Ninety-six hours after the administration of MTX the bone marrow of rats fed the NP diet showed signs of recovery, but the percentage of S-phase cells in rats fed the PD diet remained low ($P<0.01$; PD 5.2 ± 0.2, vs. NP 17.9 ± 3.6 at 96 h). This difference in bone marrow proliferation was even more enhanced 168 h after MTX administration. While no sign of recovering DNA synthesis was evident in the bone marrow of the PD group, the percentage bone marrow S-phase

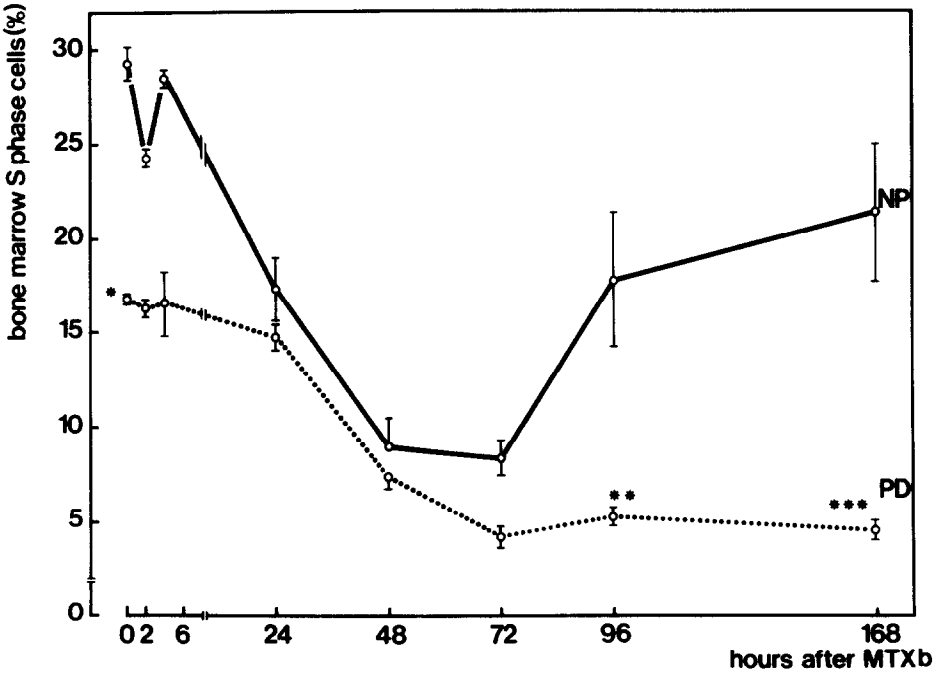

Fig. 2. The effect of MTX (30 mg/kg *body wt*) *i.p. on the percentage of S-phase cells in the bone marrow* (*mean values* ± *S.E.M.*) *from* 0 h *until* 1 *week* (168 h) *after MTX administration* (P < 0.05, **P < 0.01, ***P < 0.001).

cells of the well-nourished group reached almost normal values ($P<0.001$; PD 4.8 ± 0.2 vs. NP 21.2 ± 3.8).

Flow cytometric studies of tumor

Flow cytometry suggested that tumor tissue was insensitive to short-term protein deprivation. MTX administration, however, induced significant reductions in the percentage of S-phase cells of tumors in both PD and NP groups (Fig. 3). The tumor response to MTX seems to be slightly enhanced in the PD group compared to the NP group at 72 h post-MTX ($P<0.05$; PD control 16.1 ± 0.9 vs. PD 72 h 7.1 ± 0.9). One week (168 h) after MTX administration FCM showed that tumor DNA synthesis rate was still slightly depressed in both groups.

Intestinal morphometry

Dietary protein content *per se* did not significantly influence crypt depth and villus height of the mucosa of the small intestine. After MTX injection the mean depth of the crypts and length of the villi decreased significantly in both groups, with rats in the PD group experiencing the greatest reductions in crypt depth ($P<0.001$ at 96 h) (Fig. 4). Ninety-six hours after MTX administration the intestinal mucosa of the NP group showed the first signs of recovery, demonstrated by an increase in crypt depth. At 168 h after MTX crypt cells in both groups were recovering and showed hypertrophic crypts. Overall MTX effects on crypt depth (Fig. 4) and villus height (Fig. 5) were more pronounced and longer lasting in rats being fed the protein deprived diet as compared to rats fed the NP diet.

MTX plasma levels

The mean levels of total MTX in the plasma of rats consuming the PD diet were significantly higher at 2 and 6 h post-MTX administration than in the NP group ($P<0.05$, Table 2). Twenty-four hours after MTX administration, MTX was still measurable in the serum of PD rats, but was undetectable in the serum of rats fed the NP diet.

Tumor growth curves

Tumor growth is depicted from the day of MTX administration (day 26) onwards (Fig. 6). MTX administration induced a decrease in tumor volumes in both groups, resulting in a growth delay time of 9.5 days in the PD group and of 4 days in the NP group.

Peripheral blood

In both dietary groups significantly decreased numbers of leucocytes and platelets were measured 168 h after MTX administration, but not before (data not shown). Statistically significant differences between the groups were not present. The hemoglobin level, hematocrit and serum folinic acid levels did not change with MTX administration and showed no significant differences between the two groups during the period under study.

DISCUSSION

Weight loss is common in cancer patients and, regardless of etiology, severe tissue wasting is of major clinical significance. It is well known that malnutrition is associated with increased host toxicity to chemotherapeutic drugs in experimental

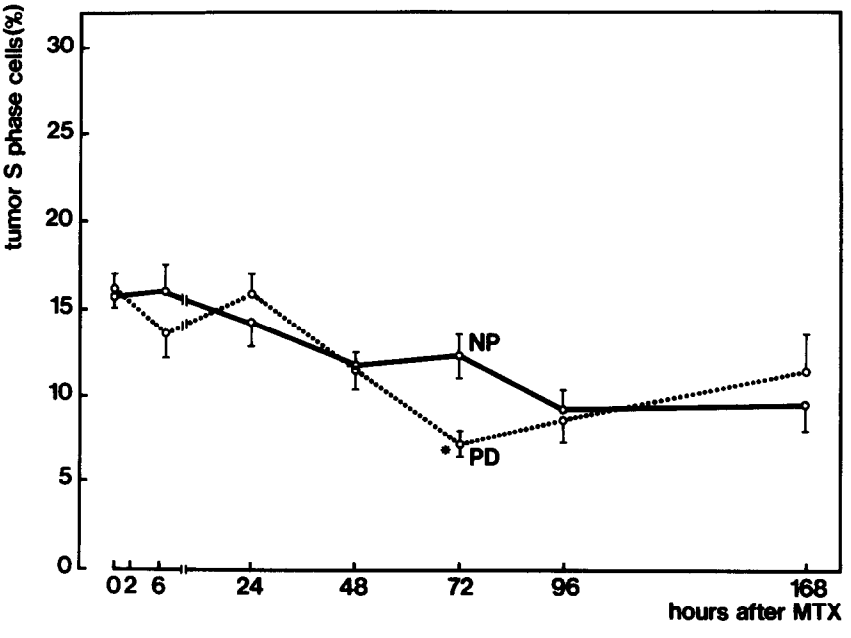

Fig. 3. The effect of MTX (30 mg/kg *body wt*) *i.p. on the percentage of S-phase cells in the tumor* (*mean values* ± *S.E.M.*) *from* 0 *h until* 1 *week* (168) *after MTX administration* (*P < 0.05).

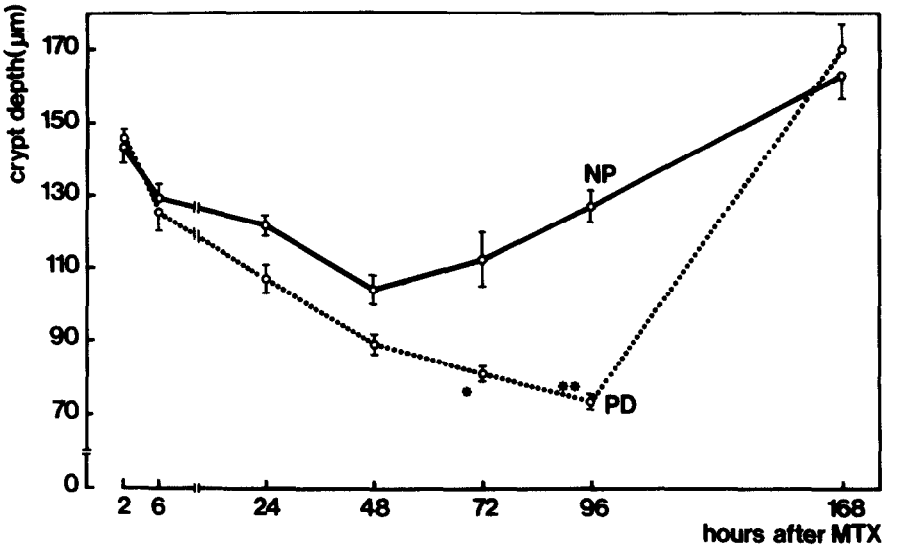

Fig. 4. The effect of MTX (30 mg/kg *body wt*) *i.p. on jejunal crypt depth* (*mean values* ± *S.E.M.*) *from* 0 h *until* 1 *week* (168 h) *after MTX administration* (*P < 0.01, *PD* 72 h: 80.0 ± 1.7 *vs. NP* 72: 112.9 ± 7.8 *and* **P < 0.001, *PD* 96 h: 73.5 ± 1.5 *vs. NP*: 128.3 ±4.8).

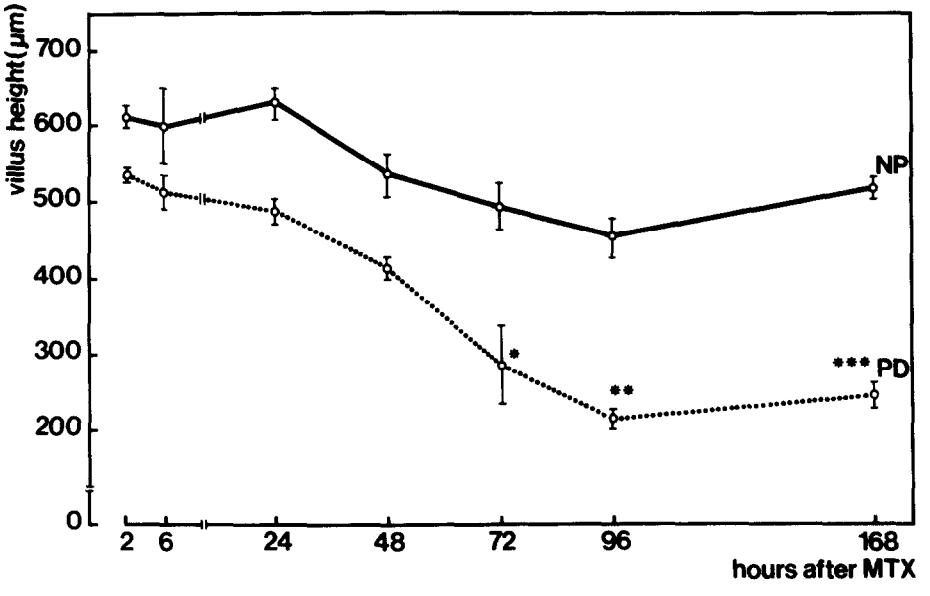

Fig. 5. The effect of MTX (30 mg/kg *body wt*) *i.p. on jejunal villus height* (*mean values* ± *S.E.M.*) *between* 0 h *and* 1 *week* (168 h) *after MTX administration* (*P< 0.001, *PD* 72 h: 278.5 ± 57.3 *vs. NP* 453.6 ± 23.1; **P < 0.001, *PD* 96 h: 211.1 ± 5.1 *vs. NP* 453.6 ± 23.1; ***P < 0.001, *PD* 168 h: 243.2 ± 14.2 *vs. NP* 544.9 ± 19.9).

cancer bearing animals and cancer patients [8–11, 17–20]. The purpose of the present study was to determine whether the host's nutritional intake influences the elimination of chemotherapeutic drugs from the body and how this affects toxicity and anti-tumor effect. The study has been executed in a rat tumor model with established growth characteristics [12, 13]. The excretion of MTX from the serum (Table 2) was delayed in PD rats resulting in a significantly enlarged area under the curve (plasma MTX concentration vs. time). Grossie *et al.* [10] reported that malnourished rats had five times the average plasma MTX levels following intravenous MTX administration as compared with control animals. This was associated with significantly lower tissue levels of cellular dihydrofolate reductase. Mihranian *et al.* [11] found plasma MTX levels to be significantly elevated 1 and 2 h post-MTX injection after feeding animals a protein-free diet for 10 days. Mean peak plasma

Table 2. Mean plasma MTX levels (nmol/l) *after i.p. administration of* 30 *mg/kg body wt in rats* (n = 16) *receiving a PD or NP diet*

Diet	Hours after MTX administration 2	6	24	48
PD	4450	126	207	<50
	3520*	166*	95	<50
NP	2360	108	<50	<50
	1260	116	<50	<50

*$P < 0.05$, PD vs. NP.

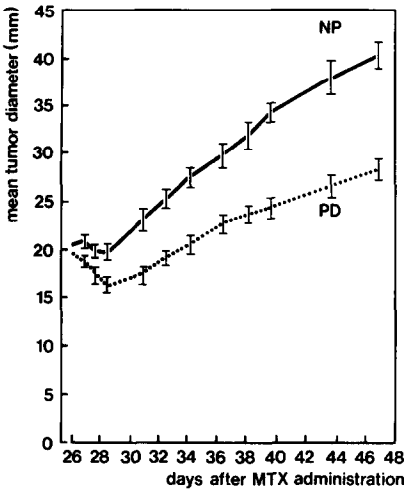

Fig. 6. Tumor growth delay is enhanced in the PD-MTX group by 5 *days compared to the NP-MTX group.*

MTX concentrations, however, were similar in malnourished and well-fed animals.

Although no objective criteria for clinical toxicity were applied it was clear from the present study that PD rats suffered more toxic side-effects of MTX than rats consuming a NP diet. Decreased food intake was more pronounced and longer lasting in the PD group and NP rats eventually recovered their pre-MTX weight, while PD rats never did.

The rapidly dividing mucosal cells of the gastro-intestinal tract and the bone marrow stem cells are sensitive to both variables used in this study, i.e. dietary manipulation and chemotherapeutic drugs. It has been shown before that dietary protein deprivation as such can significantly decrease the percentage of S-phase cells in the bone marrow [21, 22]. The deleterious effect of MTX on bone marrow proliferation was more pronounced in rats being fed a PD diet (Fig. 1). No signs of recovery were detectable in the bone marrow of PD rats during the period under study. The unfavorable influence of dietary manipulation on the gastro-intestinal mucosa can lead to reduced crypt cell proliferation and a decreased epithelial renewal rate [23–28]. Most chemotherapeutic drugs currently utilized in cancer treatment also produce morphological and functional changes in the intestinal mucosa. Morphologically this is reflected in a shortening of the crypts and blunting of villi [29–32]. In the present study decreased crypt depth and diminished villus length were manifest in both dietary groups after MTX administration. These effects were present earlier, were more pronounced and longer lasting in rats consuming the PD diet than in well-fed animals (Figs. 4 and 5).

MTX administration exerted an influence on tumor growth in both dietary groups, with tumors in the PD group experiencing a greater and longer lasting reduction in tumor volume. An earlier and more pronounced decrease in DNA synthesis seemed to be present in tumors of PD rats (Fig. 3), but it must be realized that single measurements of S-phase values as executed in the present study do not exclude a possible proportional reduction in cell cycle time [33]. In addition, changes in proliferation kinetics probably do not correspond directly with cell kill. Unfortunately the enhanced tumor response in malnourished (PD) rats is accompanied by an increased bone marrow and gastro-intestinal tract toxicity. The increased chemotherapy-related toxicity of protein-malnourished animals is in agreement with the results of Flanigan-Roat *et al.*, who showed that mortality was inversely proportional to the protein content of the diet and also demonstrated a striking delay in tumor regrowth in protein-malnourished mice [20].

The present study demonstrates alterations in serum MTX levels following dietary manipulation. Several mechanisms can explain the observed changes in MTX metabolism. Dietary manipulation may alter intestinal mass, intestinal mucosal enzyme concentrations, hepatic enzyme activity and prolong entero-hepatic circulation of MTX [34]. Malnutrition and the presence of a tumor both influence the host's metabolic state [19, 35]. MTX is cleared from the serum into the urine through an active tubular transport mechanism and therefore decreased metabolic rates, induced by malnutrition, could decrease tubular excretion rates and elevate plasma MTX levels [36]. In patients, malnutrition can induce changes in plasma protein level and plasma volume and can consequently cause changes in MTX pharmacokinetics since 50% of MTX in the serum is protein-bound [36].

The results of this study indicate that the serum clearance of MTX is delayed in malnourished rats, leading to both enhanced tumor response and increased host toxicity. It seems reasonable to suggest that a patient's nutritional state influences drug pharmacokinetics and that in calculating schemes and doses of anti-neoplastic drug administration, the nutritional state of a patient should be taken into account [6]. Two completely opposite roads for future therapy can be developed from this study.

Whether effective nutritional repletion of malnourished cancer patients would allow delivery of more adequate chemotherapy with increased response rates and prolonged survival remains to be proven [35, 37–39]. Alternatively, it seems reasonable to argue that dietary manipulation to reduce the growth of tumors would be preferential to manipulations designed to minimize the toxicity of chemotherapy, which theoretically bear the risk of tumor growth stimulation. Obviously future studies are needed to solve this problem.

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