Alteration of Chemotherapy Toxicity Using a Chemically Defined Liquid Diet in Rats

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ABSTRACT

Controversy exists as to whether administration of a chemically defined diet alters toxicity to chemotherapy. The purpose of this study was to evaluate toxicity to methotrexate in rats fed a chemically defined liquid diet or a regular chow diet. In the first study, 48 adult rats were randomized to be fed a chemically defined liquid diet or a regular diet for 14 days when methotrexate (25 or 50 mg/kg) was given. All liquid diet rats became anorexic and died within 96 h, while no deaths were observed in rats fed regular diet. When 20 liquid diet and regular diet rats were pair-fed to equalize caloric intake before and after methotrexate administration, similar mortality results occurred. In a second study, methotrexate (50 mg/kg) or saline was given to 60 h later all animals were sacrificed to obtain small bowel luminal cultures and tissue sections for histological evaluation. Administration of the liquid diet altered small bowel flora to predominantly Escherichia coli and Pseudomonas sp. and histology showed severe small bowel mucosal enteritis in comparison with regular diet rats. To evaluate whether the changes in intestinal flora or alterations in drug pharmacokinetics were responsible for the increased toxicity, two additional studies were done. Gentamicin (4.8 mg/kg/day) was given p.o. or i.m. to the rats on the chemically defined liquid diet. A significant reduction of intraluminal bacteria occurred, but survival time was not improved in animals receiving antibiotics. When mean serum methotrexate levels were analyzed in non-antibiotic-treated rats, drug concentrations were significantly increased at 24, 36, and 48 h after methotrexate injection in the elemental liquid diet rats compared with chow diet rats. Administration of a chemically defined liquid diet to rats receiving methotrexate increased the occurrence and severity of intestinal enteritis, altered intraluminal bowel flora, and decreased clearance of methotrexate from the serum.

INTRODUCTION

Both chemotherapeutic agents and diet influence the structure and function of the gastrointestinal tract. The rapidly proliferating cells of the intestinal epithelium are highly vulnerable to most chemotherapeutic agents with gastrointestinal toxicity often the limiting factor in choosing the dose of chemotherapy administered. Intestinal epithelium are also influenced by diet. Ingestion of a chemically defined diet, for example, results in changes in villous height and crypt depth as well as changes in mucosal cell turnover rates (1-3).

Controversy exists as to whether a chemically defined liquid diet alters the gastrointestinal toxicity of chemotherapeutic agents. Bounous et al. (4-6), for instance, noted a possible physiological advantage to administration of chemically defined diets to animals whose gastrointestinal tract was injured by hypoperfusion, short-lived ischemia, or the administration of an enterotoxic chemotherapeutic agent. Subsequently, the possible physiological advantage of a chemically defined diet given to animals who had received an enterotoxic chemotherapeutic agent was challenged (7-9). The purpose of this study was to determine if administration of a chemically defined liquid diet would alter the potential gastrointestinal toxicity of methotrexate.

MATERIALS AND METHODS

Several sequential studies were performed. In Study A, 48 adult male Fischer rats weighing approximately 170 g were housed in metabolic wire-bottomed cages to prevent coprophagy and were randomized to receive either a chemically defined liquid diet (n = 24) or a regular chow diet (n = 24) for 14 days. Chemically defined liquid diet animals were offered a liquid solution consisting of amino acids, glucose, and fats, as well as electrolytes, trace elements, and vitamins (Table 1). Regular diet animals were fed normal rodent chow pellets (Ralston Purina) and water (Table 1). Based on observed daily consumption patterns of chow (approximately 15 g/day) in our laboratory for this species of rat, the chemically defined diet was devised to provide comparable amounts of calories, protein, and fat, as well as required vitamins, trace elements, and electrolytes. The chemically defined liquid diet provided 1.3 KCal/ml and 0.064 g protein/ml. The chow diet provided 4.5 KCal/g and 0.23 g protein/g of chow consumed. Each diet provided comparable amounts of folate.

All animals were allowed to eat and drink ad libitum. Fresh solution and pellets were offered each day, and the amount consumed was recorded. All animals were weighed on days 0, 5, 9, 12, and 14. On day 14, the rats were randomized and given i.p. injections of methotrexate, 25 (n = 24) or 50 (n = 24) mg/kg. All groups of animals were continued on their prescribed diets after the administration of methotrexate. All rats were observed twice daily for evidence of toxicity. Autopsies were performed on all animals that died. Animals that survived were subsequently followed to observe possible late signs of drug toxicity.

Using an identical protocol, 20 adult male rats were randomized to receive either a chemically defined liquid diet (n = 10) or regular rat chow (n = 10) for 14 days. Regular chow animals were pair-fed to receive the amount of calories ingested by the chemically defined liquid diet rats. On day 14, all rats received methotrexate, 25 mg/kg i.p. Pair-feeding continued after methotrexate administration and rats were observed twice daily for signs of toxicity.

In Study B, 24 adult male Fischer rats were housed in metabolic cages and fed ad libitum the same chemically defined diet (n = 12) or regular diet (n = 12). The amount of solution or pellets consumed and body weight were recorded in the same fashion as in Study A. On day 14, methotrexate, 50 mg/kg, was given i.p. to six liquid diet rats and six regular diet rats. The higher dose was selected to maximize possible toxic manifestations. Five chemically defined liquid diet rats and six regular diet rats were given saline injections. Sixty h after injection with methotrexate or saline, the animals were anesthetized with ether, and a laparotomy was performed under sterile conditions. Inferior vena cava and portal vein blood samples were drawn for aerobic and anaerobic cultures. A 1.5-cm segment of distal small bowel was then isolated between clamps and treated by injection of 1 ml of sterile saline which was then mixed with the intraluminal contents of the bowel. Fluid was then aspirated for aerobic and anaerobic cultures. Separate segments of distal small bowel and proximal colon were then harvested and fixed in formalin for later staining with hematoxylin and eosin.

Cultures were done on a semiquantitative basis. For each animal, 1 ml each of portal vein and inferior vena cava blood and 1 ml of aspirated small bowel contents, respectively, were divided equally and plated on two separate agar plates (Columbia Blood Agar Plates) for aerobic and anaerobic cultures. Aerobic incubation took place at 35°C under a 5% CO₂ atmosphere. Anaerobic cultures were incubated at 35°C under...
anaerobic conditions. Aerobic and anaerobic plates were assessed and counted at 48 and 72 h after plating. Seventy-two-hour results are reported. Conventional methods were used for identifying Gram-positive organisms. Coliform species and Pseudomonas sp. were identified using the Microscan Identities System.

In Study C (Fig. 1), 120 adult male Fischer rats weighing 180–200 g were fed ad libitum either a chemically defined liquid diet or a regular diet (n = 40). On day 5, chemically defined liquid diet rats were given gentamicin, 4.8 mg/kg/day p.o. (n = 20), or i.m. (n = 20). Gentamicin was chosen for administration because the Escherichia coli and Pseudomonas consistently cultured from the small bowel lumen of rats, though, were able to maintain pre-methotrexate food consumption of both groups of animals are shown in Table 2. Chemically defined liquid diet animals consumed 97% as many calories per day and 91% as much protein per day as regular diet animals. The difference reflects the slight difference in the calorie/nitrogen ratio in the different diets. After administration of methotrexate, chemically defined liquid diet animals consumed progressively fewer calories and protein. Regular diet rats, though, were able to maintain pre-methotrexate food consumption patterns in the immediate 3-day period after administration of methotrexate (Table 2).

Both groups of animals were well matched with respect to initial body weight (172 ±2 versus 167 ±3 g) and gained comparable amounts of weight during the 14 days before receiving methotrexate (Fig. 2). After administration of methotrexate, chemically defined liquid diet animals lost weight, while regular diet animals maintained their body weight.

RESULTS

Study A

Food Consumption and Body Weight. Calorie and protein consumption of both groups of animals are shown in Table 2. Chemically defined liquid diet animals consumed 97% as many calories per day and 91% as much protein per day as regular diet animals. The difference reflects the slight difference in the calorie/nitrogen ratio in the different diets. After administration of methotrexate, chemically defined liquid diet animals consumed progressively fewer calories and protein. Regular diet rats, though, were able to maintain pre-methotrexate food consumption patterns in the immediate 3-day period after administration of methotrexate (Table 2).

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In Study D, 70 adult male Fischer rats weighing approximately 200 g were randomized to receive either a chemically defined liquid diet or regular chow diet for 7 days. After administration of methotrexate, 20 mg/kg i.p., five rats from each group were sacrificed at 1, 2, 6, 12, 24, 36, and 48 h and blood samples were taken for methotrexate analysis. Blood samples were obtained by cardiac puncture and were immediately centrifuged. Serum was then separated and frozen. The methotrexate assay was performed by placing 20-ml aliquots of serum on filter paper discs and allowing them to air dry. These discs were then placed on uniform lawns of Streptococcus faecium and the plates were placed in a 38°C incubator. After 18 h, the circle of bacterial inhibition was measured and results were analyzed by comparison with a standard inhibition curve. All measurements were carried out in duplicate and the inhibition curve was restandardized for each day's analysis. This assay is capable of measuring accurately methotrexate levels of 0.5 ng/ml of sample.

All values are expressed as mean ± SE. Statistical analysis was performed using a nonpaired Student t test, and analysis of variance was used to evaluate methotrexate concentrations over time.

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Table 2 Food consumption

<table>
<thead>
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<th></th>
<th>Chemically defined liquid diet</th>
<th>Regular chow diet</th>
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<tbody>
<tr>
<td></td>
<td>((n = 24))</td>
<td>((n = 24))</td>
</tr>
<tr>
<td>Study A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-methotrexate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calories (kcal/day)</td>
<td>(67 \pm 3^a)</td>
<td>(69 \pm 4)</td>
</tr>
<tr>
<td>Protein (g/day)</td>
<td>(3.4 \pm 0.1)</td>
<td>(3.7 \pm 0.6)</td>
</tr>
<tr>
<td>Study B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-methotrexate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calories (kcal/day)</td>
<td>(83 \pm 4)</td>
<td>(77 \pm 3)</td>
</tr>
<tr>
<td>Protein (g/day)</td>
<td>(4.1 \pm 0.2)</td>
<td>(4.1 \pm 0.2)</td>
</tr>
</tbody>
</table>

\(^a\) Mean ± SE.

\(\text{MTX/saline}\)

Fig. 3. Mean body weight gain in animals in Study B given a chemically defined liquid diet \((\text{CDD})\) compared with a regular chow diet \((\text{RD})\). MTX, methotrexate.

Survival. All liquid diet animals died within 60–96 h after administration of methotrexate at 25 or 50 mg/kg. These animals stopped eating by the third day after methotrexate administration, developed diarrhea, became increasingly lethargic, and died. No regular diet animals died immediately after methotrexate administration or in the ensuing 2-week period.

Using the identical protocol, 10 regular diet rats were pair-fed to 10 rats receiving the chemically defined liquid diet for 14 days. Initial and day 14 mean body weights were 231 ± 24 and 232 ± 24 g in the liquid diet group and 243 ± 25 and 246 ± 26 g, respectively, in the regular diet group. Pair-feeding continued after methotrexate administration at 5–10 kcal/day. Nine of the chemically defined liquid diet rats were dead by 96 h after methotrexate administration (90% mortality), while all regular diet animals survived beyond 7 days.

Autopsy Findings. All 24 liquid diet animals in Study A were autopsied and gross pathological changes were noted. One animal showed evidence of colonic perforation that resulted from the i.p. injection. No other animals showed signs of viscus perforation. There was no exudate or purulent material in the peritoneal cavity. The small bowel and colon appeared attenuated and almost translucent. There was evidence of intraluminal blood with pooling in the cecum. The lining of the gastrointestinal tract showed varying degrees of focal necrosis and sloughing. There was only a minimal amount of stool in the colon. The remainder of the intraabdominal organs were grossly normal.

Study B

Food Consumption and Body Weight. Calorie and protein consumption of chemically defined liquid diet rats averaged 83 ± 4 kcal/day and 4.1 ± 0.2 g/day. Regular diet rats ingested an average of 77 ± 3 kcal/day and 4.1 ± 0.2 g protein/day, respectively. After administration of saline, regular diet and chemically defined diet animals maintained their normal pattern of oral consumption. Chemically defined liquid diet animals who received methotrexate maintained their normal intake in the first 2 days following methotrexate administration, but then oral intake decreased precipitously (Table 2). In the first 2 days after methotrexate administration chemically defined liquid diet animals consumed more than their regular diet counterparts.

Both dietary groups of rats were well matched with respect to mean initial body weight. The animals gained comparable amounts of weight in the 14-day pre-methotrexate period (Fig. 3). One rat in the chemically defined liquid diet that was not to receive methotrexate, however, died of unexplained causes on day 10 of the premethotrexate period.

Blood Cultures. In the chemically defined liquid diet rats receiving methotrexate, there was no bacterial growth from either portal vein or inferior vena cava blood cultures. In the chemically defined liquid diet animals not receiving methotrexate two of five portal vein blood cultures were positive, but none of the inferior vena cava blood cultures showed bacterial growth. Regular diet rats receiving methotrexate had a single positive portal vein blood culture, while regular diet rats not receiving methotrexate had two positive portal vein and a single positive
inhibit vena caval blood culture. No animal had both positive portal vein and inferior vena caval blood cultures. All blood cultures grew approximately 10 to 15 colonies of *Lactobacillus* sp. per plate.

**Small Bowel Cultures.** Whether the animals were given methotrexate or not, small bowel content cultures of regular diet animals were similar (Table 3). Predominant species grown were *Lactobacillus* and nonhemolytic *Streptococcus*. These animals also grew varying numbers of *Bacillus* colonies. One rat in each group grew a few *Klebsiella* colonies, while one regular diet rat who was not given methotrexate grew a small quantity of *Citrobacter* colonies. There was no growth of *E. coli* or *Pseudomonas* sp. in the animals fed a regular chow diet.

Chemically defined liquid diet rats, whether or not they received methotrexate, grew *E. coli* as their predominant species (Table 3). In only one liquid diet animal was there no growth of *E. coli*. *Pseudomonas* was found in two of six liquid diet rats receiving methotrexate and three of five liquid diet rats not receiving methotrexate. Varying numbers of *Bacillus* and *Lactobacillus* colonies were seen in all the animals fed a chemically defined liquid diet. Anaerobic species were not cultured in these studies because either their number was few or their growth was overshadowed by aerobic organisms that were able to grow well under anaerobic conditions.

**Histology.** Small bowel sections from chemically defined liquid diet rats who received methotrexate demonstrated severe enteritis (Fig. 4). The mucosa was thin and villi were flattened. Multiple mucosal erosions and areas of focal hemorrhage were noted. The crypts were infiltrated with polymorphonuclear cells, and the inflammation extended through the muscularis mucosae. The colon of these animals demonstrated very mild erosions of the mucosa with disruption of the normal villous architecture. Small areas of focal inflammation were seen in the crypts.

The small bowel of regular diet animals receiving methotrexate showed minimal changes in relation to the chemically defined liquid diet animals receiving methotrexate. A few villi were flattened or showed minimal disruption of their normal architecture. Erosions and areas of focal hemorrhage were not present, and the inflammatory response in the crypts was much less than that seen in the chemically defined liquid diet animals receiving methotrexate. Regular diet and chemically defined liquid diet rats who were not given methotrexate were compared to determine if the type of diet given altered the basic histological structure of small bowel and colon. Slides of small bowel and colon from both groups of animals were reviewed blindly, and no major differences in mucosal structure could be discerned between the animals that were fed the different diets for 17 days.

**Study C**

All regular diet rats alive at least 8 days after methotrexate administration without evidence of toxicity. Their survival time was determined as 192 h and was significantly longer than the survival time in all the rats fed the chemically defined liquid diet. In the chemically defined diet groups, mean survival time was 111 ± 15 h in the i.m. gentamicin group compared with 114 ± 19 h in its corresponding i.m. saline control group (not significant; Fig. 5). Survival patterns in rats receiving gentamicin p.o. was not significantly improved in comparison with its control group (Fig. 6).

There was no significant difference in mean small bowel bacterial content in the i.m. gentamicin group compared with the corresponding saline control group (272 ± 121 versus 147 ± 83 colonies). However, the mean small bowel colony count was significantly lower in the p.o. gentamicin group compared with the corresponding p.o. control group (104 ± 78 versus 383 ± 110 colonies; *P < 0.01*). One of five rats in the p.o. gentamicin group had no bacterial growth from cultures of small bowel content.

**Study D**

No statistically significant differences in mean serum methotrexate levels were noted between groups in the initial (1 to 12 h) uptake of methotrexate from the peritoneal cavity and its clearance from the serum. At 24, 36, and 48 h there was a significant (*P < 0.03*) increase in methotrexate concentration in the liquid diet group compared with the chow diet group as shown in Table 4.

### DISCUSSION

Bounous et al. (4–6) first noted the possible value of a chemically defined liquid diet in protecting intestinal epithelium against damage produced by hypoperfusion, short-lived ischemia, and the administration of 5-FU.2 Gardner et al. (7, 8) subsequently challenged the findings of Bounous. They found that water and glucose absorption was impaired after 5-FU administration and was independent of diet. Likewise, they found that cytoplasmic peptide hydrolase activities and mucosal DNA content were decreased after giving 5-FU, and the use of a chemically defined liquid diet did not protect the animals from developing these biochemical and tissue deficiencies. In fact, Stanford et al. (9) gave 5-FU to animals on a chow diet and various elemental diets and observed greater mortality in animals fed an elemental diet versus those animals fed a control chow diet. The differences were not dramatic nor did the authors provide statistical analysis of their findings.

While Stanford et al. suggested that chemically defined liquid diets increased the toxicity of an enterotoxic chemotherapeutic agent (5-FU), our studies showed that feeding a chemically defined liquid diet prior to and during administration of methotrexate was highly lethal. Our 100% mortality resulted from a dose of methotrexate that was well below the 50% lethal dose

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Methotrexate (n = 6)</th>
<th>No methotrexate (n = 6)</th>
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<tbody>
<tr>
<td></td>
<td>No. of rats with</td>
<td>Av. no. of</td>
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<tr>
<td></td>
<td>positive cultures</td>
<td>colonies/plate*</td>
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<tr>
<td>Lactobacillus</td>
<td>6</td>
<td>&gt;400</td>
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<tr>
<td>Nonhemolytic</td>
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<td>275</td>
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<tr>
<td>Streptococcus</td>
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<td>40</td>
</tr>
<tr>
<td>Bacillus</td>
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<td>3</td>
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<td>Klebsiella</td>
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<td>Lactobacillus</td>
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<td>40</td>
</tr>
<tr>
<td>Pseudomonas</td>
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<td>50</td>
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</table>

* Each plate cultured with 0.5 ml of aspirated small bowel content.

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2 The abbreviation used is: 5-FU, 5-fluorouracil.
Fig. 4. Histological section of small intestine from animals given a chemically defined liquid and methotrexate (50 mg/kg) shows marked mucosal and submucosal inflammatory changes with loss of normal villus architecture.

Fig. 5. Survival patterns in rats given a chemically defined liquid diet with (●) or without (○) gentamicin, 4.8 mg/kg i.m. N.S., not significant; MTX, methotrexate.

Fig. 6. Survival patterns in rats given a chemically defined liquid diet with (●) or without (○) gentamicin, 4.8 mg/kg. N.S., not significant; MTX, methotrexate.

(>125 mg/kg) of methotrexate that we determined in our laboratory for this species of rat ingesting regular chow diet. The rapidity with which this toxicity became manifest is of major interest. Chemically defined liquid diet rats ate for 24 to 48 h after receiving a single dose of methotrexate. After this they became anorexic, developed diarrhea, and died. Anorexia was not the cause of death in these animals; we had noted similar mortality when chemically defined liquid diet and regular diet animals were pair-fed after administration of methotrexate.

The chemically defined liquid diet used in this experiment provided adequate amounts of nutrients to sustain animal growth without complications. Diets similar to ours have been used by other investigators with comparable results (1, 10). There were no significant differences in the amounts of protein and calories that liquid diet and regular diet animals received in any of these studies prior to methotrexate administration. Weight gain in the period prior to giving methotrexate was comparable when comparing both groups of animals in all studies.

Bacterial flora of the gastrointestinal tract changed in animals fed a chemically defined liquid diet. These animals showed a greater growth of coliform species in comparison to regular diet animals where Lactobacillus sp. and nonhemolytic Streptococcus dominated. We cultured the chemically defined liquid diet solution and found that the changes in flora were not due to colonization of the gastrointestinal tract from contamination of this solution. The solution was prepared in a sterile fashion and changed daily. At 24 h remaining solution grew only a few Lactobacillus colonies. While the chemically defined liquid diet changed the intraluminal milieu that led to growth of different bacterial species in these studies, no consistent pattern of microbiological changes induced by chemically defined liquid diet has emerged (11, 12).

Certainly, the growth of coliform species in our liquid diet animals provided a more dangerous environment that the more benign Lactobacillus found in regular diet animals. If bactere-
mia were more likely in methotrexate-treated animals because of the substantial enteritis that developed, then the host gastrointestinal bacterial populations of the liquid diet animals would be more potentially lethal than the endogenous bacterial population of the regular diet animals.

Stanford et al. (9) noted an increase in positive blood cultures in elemental diet animals versus regular diet animals that received 5-FU. Interestingly, the degree of hydrolysis of protein in the diet correlated with the occurrence of positive cultures in their study. Our chemically defined diet provided completely hydrolyzed protein. However, we were not able to demonstrate significant amounts of bacteria in the portal vein or inferior vena cava in animals receiving both a chemically defined diet and methotrexate. All positive blood cultures occurred in other groups of animals and grew Lactobacillus sp. and not more virulent, invasive bacteria.

Study C was designed to investigate the possibility that alteration of gastrointestinal tract flora was the cause of increased toxicity and mortality in chemically defined liquid diet rats. In vitro testing revealed that E. coli, Pseudomonas sp., Lactobacillus sp, and Bacillus sp. were sensitive to gentamicin, which was thus chosen for study. However, administration of gentamicin either enterally or parenterally did not improve mean animal survival time compared with length of survival of rats in regular diet animals. Diminution of bacterial growth was demonstrated in the small bowel cultures from p.o. gentamicin rats compared with p.o. control rats. However, neither parenteral gentamicin in a dosage sufficient to treat sepsis nor p.o. gentamicin in a dosage sufficient to lower intestinal bacterial count had any effect on mean survival time.

What is evident from autopsy results in Study A and the histological assessment from Study B is that the chemically defined liquid diet animals developed severe inflammation and destruction of the small bowel mucosa after administration of methotrexate. Subsequent dehydration and intraluminal bowel hemorrhage led to the death of these animals. Decreased p.o. intake after administration of methotrexate in the liquid diet rats probably resulted from severe gastrointestinal enteritis. The high mortality observed in these rats occurred because of a change in the enterotoxic potential of methotrexate in animals fed a chemically defined diet. Supporting this contention is the histological assessment from Study B is that the chemically defined liquid diet animals developed severe inflammation and destruction of the small bowel mucosa after administration of methotrexate.

Diet is known to alter the pharmacokinetics of many drugs. Methotrexate toxicity is directly related to a tissue's duration of exposure to the drug once a critical toxic threshold is reached. Elimination of methotrexate depends on its excretion in the bile and urine (13, 14). It is known that there is a significant enterohepatic circulation of the drug. Study D was performed to determine if the chemically defined liquid diet altered the pharmacokinetics of the drug and increased its enterotoxic potential. Elevations in serum methotrexate levels were demonstrated in rats fed the chemically defined liquid diet when compared with Chow diet-fed rats. In the Chow-fed rats methotrexate was virtually cleared from serum within 24 h of injection while in the chemically defined liquid diet rats significant levels of methotrexate persisted for at least 48 h. As stated above methotrexate toxicity is directly related to the tissue levels over time. These studies give the first clear evidence that the cause of increased methotrexate toxicity seen in chemically defined diet fed rats may be secondary to decreased clearance of methotrexate from the serum.

REFERENCES

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