Therapeutic Efficacy of Cyclophosphamide as a Function of Its Metabolism

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SUMMARY

Experiments were designed to investigate the metabolism of cyclophosphamide in vitro and in vivo following the administration of known stimulators and depressors of rat hepatic microsomal mixed-function oxidase activity, the antitumor efficacy of cyclophosphamide as a function of its metabolism, and the toxicity of cyclophosphamide as a function of its metabolism. Regarding the metabolism of cyclophosphamide by hepatic microsomal preparations, the following observations were made. (a) Pretreatment with phenobarbital of male and female rats and of female mice increased the rate of metabolism 7-, 23-, and 7-fold, respectively. (b) Pretreatment of male rats with 3-methylcholanthrene depressed metabolism to 33% of that of controls, but similar pretreatment of female mice did not alter the rate of metabolism. (c) The K_m for cyclophosphamide metabolism by microsomes obtained from male rat liver changed from 1.39 mM to 0.57 and 0.58 mM after phenobarbital and 3-methylcholanthrene pretreatment, respectively. (d) Pretreatment of male rats with thioacetamide, morphine, or cobalt chloride depressed metabolism to 12, 48, and 8% of control, respectively. (e) Male rats bearing the Walker 256 carcinosarcoma i.m. showed a depressed ability to metabolize cyclophosphamide. In vivo cyclophosphamide metabolism in rats paralleled in vitro metabolism. Thus, at early time points, blood levels of alkylating activity were (a) increased following pretreatment with phenobarbital, (b) decreased following pretreatment with 3-methylcholanthrene or cobalt chloride, (c) decreased when the animal bore the Walker 256 carcinosarcoma i.m., and (d) lower in female compared with male rats. Walker 256 carcinosarcoma cells grown i.m. in the hindlegs of male and female rats were used to evaluate therapeutic efficacy. The dose of cyclophosphamide that inhibits tumor growth in male rats by 50% was 0.7 mg/kg. Similar median effective doses were obtained in female rats and in male rats pretreated with phenobarbital or cobalt chloride. 3-Methylcholanthrene pretreatment increased the median effective dose of cyclophosphamide to 3.5 mg/kg. For estimation of the toxicity of cyclophosphamide, blood leukocyte counts were made at various intervals following injection of cyclophosphamide. Little difference in the decline of the number of leukocytes or in their subsequent return to normal levels was observed between control male rats; female rats; and phenobarbital-, 3-methylcholanthrene-, or cobalt chloride-pretreated male rats. Pretreatment with phenobarbital did accelerate leukocyte depression and also increased the magnitude of depression. In methylcholanthrene-pretreated male rats and in female rats, the magnitude of depression was somewhat less and recovery rates were somewhat altered. Administered by itself, phenobarbital, 3-methylcholanthrene, or cobalt chloride had no effect on tumor growth or blood leukocyte levels. The data demonstrate the futility of trying to improve the therapeutic efficacy of cyclophosphamide by pretreatment with drugs that alter its rate of activation. In addition, the data provide a rational basis for the ineffectiveness of such an effort.

INTRODUCTION

Cyclophosphamide is an antineoplastic agent thought to exert its therapeutic effect via alkylation. However, cyclophosphamide itself exerts minimal alkylating activity, does not exert a cytostatic effect in vitro, and must be activated in vivo to exert its biological effect. Reports from this and other laboratories indicate that the activation of cyclophosphamide is catalyzed by the same hepatic microsomal mixed-function oxidases that are functional in the oxidative metabolism of many other drugs (2, 4, 5, 7, 8, 26). With the knowledge that cyclophosphamide is activated by hepatic microsomal mixed-function oxidases and with the wealth of information available as to the stimulation and depression of these enzyme activities (21), experiments can be readily designed to regulate the metabolism of cyclophosphamide. Thus, a commonly used inhibitor of hepatic microsomal mixed-function oxidase activity, SKF 525A, inhibits the conversion of cyclophosphamide in vivo to its alkylating and cytotoxic metabolite(s) (4), and pretreatment with phenobarbital, a well-known inducer of these enzymes, causes a marked increase in serum levels of alkylation activity after cyclophosphamide administration (2, 23). However, neither SKF 525A nor phenobarbital pretreatment had an effect on the antitumor (leukemia L1210) efficacy of cyclophosphamide (17), although SKF 525A, 2,4-dichloro-6-phenylphenoxethyl-diethylamine, and chloramphenicol, all inhibitors of hepatic microsomal mixed-function oxidase

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2 The abbreviations used are: SKF 525A, 2-diethylaminoethyl-2,2-diphenylvalerate; nor-HN2, bis(2-chloroethyl)amine hydrochloride; ED_50, dose of cyclophosphamide (in mg/kg) that inhibits tumor growth 50%.
activity, did reduce the lethality of cyclophosphamide to mice and rats (11, 17), while pretreatment with phenobarbital hastened the onset of lethality (7, 17). In contrast, pretreatment with phenobarbital virtually abolishes the teratogenic effect of cyclophosphamide, whereas the administration of SKF 525A enhances it (14).

Experiments described in this paper were undertaken to determine (a) the kinetic characteristics of hepatic microsomal cyclophosphamide metabolism following the administration of drugs known to increase or decrease hepatic microsomal mixed-function oxidase activity and thought to do so by increasing or decreasing the amount of enzyme present, (b) whether the administration of drugs that stimulate or depress the metabolism of cyclophosphamide in vivo do so in vitro, (c) whether the antitumor efficacy of cyclophosphamide against an experimental tumor system (Walker 256 carcinosarcoma) is a function of its rate of metabolism, and (d) whether the undesired toxicity of cyclophosphamide is a function of its rate of metabolism.

MATERIALS AND METHODS

Animals and Dosage Schedules. Male and female Holtzman rats (230 to 270 g) obtained from Holtzman Laboratories, Madison, Wis., and female Swiss-Webster mice (23 to 27 g) obtained from Simonsen Laboratories, White Bear, Minn., were used in all experiments except those in which 170- to 180-g male and female Holtzman rats were given i.m. injections of Walker 256 carcinosarcoma ascites cells. In all experiments the animals were fed a standard chow diet ad libitum.

Phenobarbital sodium (Merck & Co., Inc., Rahway, N. J.), 40 mg/kg, was injected i.p. in a volume of about 0.5 ml of 0.9% NaCl solution every 24 hr (into rats) or every 12 hr (into mice) for 5 days before sacrifice or injection of cyclophosphamide. Thioacetamide (Fisher Scientific Co., Pittsburgh, Pa.), 50 mg/kg, or morphine sulfate (Merck), 20 mg/kg, were injected i.p. in a volume of about 0.5 ml of 0.9% NaCl solution at 96, 72, 48, and 24 hr before sacrifice. 3-Methylcholanthrene (Eastman Organic Chemicals, Rochester, N. Y.), 20 mg/kg, was injected i.p. in a volume of about 0.5 ml of corn oil at 72 and 48 hr before sacrifice or injection of cyclophosphamide. Cobalt chloride (J. T. Baker Chemical Co., Phillipsburg, N. J.), 90 mg/kg, was injected s.c. in a volume of 0.15 ml of 0.9% NaCl solution at 48 and 24 hr before sacrifice or injection of cyclophosphamide. Preliminary studies revealed that maximum effects on rat hepatic microsomal mixed-function oxidase activity are achieved with the dosage schedules described. Control animals received the appropriate volume of vehicle by the appropriate route of administration. Preliminary studies revealed that these drugs, in the dosage schedules used, had no effect on tumor weights determined 7 days after the introduction of 1 million Walker 256 carcinosarcoma cells, ascites form, into the hindleg muscles of rats.

Tissue Preparation and Estimate of Cyclophosphamide Metabolism in Vivo. All animals were sacrificed between 8 and 9 a.m. Hepatic microsomal preparations were obtained as previously described (26).

The incubation mixture and the colorimetric estimation of metabolites capable of alkylation were as described previously (26), except that semicarbazide hydrochloride (37.5 μmoles/reaction flask) was used in all experiments, and microsomes obtained from 50 mg of wet liver were used in each reaction flask when phenobarbital-treated animals were the source of liver.

Estimate of Cyclophosphamide Metabolism in Vivo. Untreated, drug-treated, or vehicle-treated rats were given injections i.p. of cyclophosphamide, 400 mg/kg, in a volume of 2.6 ml. Blood samples (0.6 ml) were collected from the tail vein into a heparinized pipet at 15, 30, 60, 120, 180, and 1440 min after the introduction of cyclophosphamide. The blood samples were placed into centrifuge tubes containing 5.4 ml of 0.01 M phosphate buffer, pH 7.4. Two ml of a 5.5% ZnSO4·7H2O solution were added, followed by 2 ml of a 4.5% Ba(OH)2·8H2O solution. After centrifugation, 3 ml of the supernatant were transferred to screw-capped test tubes containing 1 ml of a 0.2 M acetate buffer, pH 4.0, and alkylating activity was determined as described previously (26). The remainder of the supernatant was extracted with 15 ml of methylene chloride by shaking for 30 min in an Eberbach shaker unit at approximately 125 excusions per min. Following centrifugation to aid the separation of phases, 3 ml of the aqueous phase were assayed for alkylating activity as described above. Alkylating activity present in blood was expressed in terms of nor-HN2 equivalents (26).

Estimate of Tumor Growth Rate. One million Walker 256 carcinosarcoma cells, ascites form (obtained from Cancer Chemotherapy National Service Center via Arthur D. Little, Inc., Cambridge, Mass.), in a volume of 0.1 ml, were injected into hind leg muscle, by means of techniques suggested by the Cancer Chemotherapy National Service Center (6). At various times thereafter, the tumor-bearing hind leg and the contralateral leg were cut off at the head of the femur. The difference in weight was taken as an estimate of tumor weight.

Dose Response and ED50 Determinations. One million Walker 256 carcinosarcoma cells, ascites form, in a volume of 0.1 ml were injected i.m. into the hindlegs of control or pretreated rats. Twenty-four hr later, freshly prepared cyclophosphamide in 0.9% NaCl solution was injected i.p. at dosage levels of 0, 0.25, 0.5, 1.0, 2.0, and, in some experiments, 4.0 mg/kg. Four to 6 rats were used at each dosage level. Tumor growth and inhibition thereof were determined 7 days after the introduction of tumor cells by the methods described above. All tumor weights in the experimental group comprised of tumor-bearing rats treated with cyclophosphamide were expressed as percentage of controls, i.e., tumor-bearing rats not treated with cyclophosphamide, in the dose response and ED50 determinations.

Estimate of Cyclophosphamide Toxicity. Except to establish a dose-response curve, 50 mg of freshly prepared cyclophosphamide per kg in 0.9% NaCl solution were routinely injected i.p. Blood was obtained from the tail vein at 48 and 24 hr determined 7 days after the introduction of 1 million Walker 256 carcinosarcoma cells, ascites form, into the hindleg muscles of rats.
various times thereafter, and leukocyte counts were determined by means of standard techniques (9).

**Data Analyses.** All data pertaining to reaction velocities were evaluated as previously described (26). Data describing dose-response curves were analyzed by the parallel line assay of Finney (13), with the aid of a CDC 3300 computer (Control Data Corp., Minneapolis, Minn.).

**RESULTS**

More than 200 drugs and other compounds are known to stimulate hepatic microsomal mixed-function oxidase activity (21). Two of the more commonly used inducers are phenobarbital and 3-methylcholanthrene. Phenobarbital is one of many compounds that are relatively nonspecific, in that they stimulate the metabolism of many drugs. These compounds are thought to stimulate the synthesis of the normally occurring microsomal hemoprotein, cytochrome P-450; this cytochrome is believed to be relatively nonspecific in that it participates in the oxidation of many substrates. 3-Methylcholanthrene is one of a relatively small number of compounds, mostly polycyclic hydrocarbons, that are somewhat specific, in that they stimulate the metabolism of only a few drugs and decrease the metabolism of others. The polycyclic hydrocarbons are thought to effect increased metabolism by stimulating the synthesis of a microsomal hemoprotein, cytochrome P-1,450, which is undetectable in untreated rats and which can function in the metabolism of only a small number of substrates (27—29).

Following pretreatment with phenobarbital, increased metabolism of cyclophosphamide, in vivo and in vitro, has been observed in several species (2, 7, 22, 23). Stimulation of cyclophosphamide metabolism in vitro by phenobarbital pretreatment has been confirmed and extended in the present experiments to include Michaelis-Menten kinetic constants (Table 1). Administration of phenobarbital increased $V_{\text{max}}$ about 7-fold in male rats and female mice and about 20-fold in female rats. Of interest is the finding that the $K_m$ for this reaction changes after administration of phenobarbital to male rats but does not after the administration of the barbiturate to female rats or mice. At present, the interpretation and significance of this observation are not clear.

3-Methylcholanthrene pretreatment of male rats failed to increase microsomal metabolism of cyclophosphamide; in fact, both $V_{\text{max}}$ and $K_m$ were decreased (Table 1). A previous communication provided evidence for the participation of cytochrome P-450 in the metabolism of cyclophosphamide and for the classification of cyclophosphamide as a type 1 compound (26). The present experiments indicate that cytochrome P-1,450 is unable to participate in the metabolism of cyclophosphamide. Metabolism of type 1 compounds is known to be stimulated by phenobarbital pretreatment but not by polycyclic hydrocarbon pretreatment. Thus, the current observations provide more evidence for the classification of cyclophosphamide as a type 1 compound. The interpretation and significance of the change in $K_m$ for cyclophosphamide metabolism following 3-methylcholanthrene administration to male rats is not clear.

Pretreatment of female mice with 3-methylcholanthrene did not alter the kinetic parameters examined (Table 1), which is not surprising, since 3-methylcholanthrene does not cause the formation of cytochrome P-1,450 in female mice of the strain used (N. E. Sladek, unpublished observations).

The administration of thioacetamide, morphine, or cobalt chloride to adult male rats is known to depress the metabolism of type 1 compounds by hepatic microsomal mixed-function oxidases (18, 28, 31), presumably by inhibiting synthesis of the enzyme. All of these compounds depressed the in vitro metabolism of cyclophosphamide (Table 2).

The question arose as to whether the increases or decreases in metabolism observed in vitro could be effected in vivo. Cyclophosphamide was injected into untreated male and female rats and into phenobarbital-, 3-methylcholanthrene-, or cobalt chloride-pretreated male rats. Blood samples were taken at various times thereafter, and total alkylating activity (Chart 1) and alkylating activity remaining after methylene chloride extraction (Chart 2) were determined. The latter was determined because, under the conditions of our assay, the parent compound does possess some alkylating activity, about 2.5% of that of nor-HN2 on a molar basis. Because high doses of cyclophosphamide were injected and because only a small part of the parent compound would be expected to be metabolized in the early time periods under consideration, much of the total alkylating activity observed could be due to cyclophosphamide itself. Methylene chloride has been reported to extract cyclophosphamide from aqueous solutions.

### Table 1

**Kinetics of cyclophosphamide metabolism by hepatic microsomes from saline-, phenobarbital-, and 3-methylcholanthrene-pretreated rats and mice**

<table>
<thead>
<tr>
<th>Source</th>
<th>Treatment</th>
<th>$V_{\text{max}}$ (µmoles/g/hr)</th>
<th>$K_m$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male rats</td>
<td>0.9% NaCl solution</td>
<td>4.20 ± 0.38</td>
<td>1.39 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>Phenobarbital</td>
<td>30.06 ± 0.86</td>
<td>0.57 ± 0.03^a</td>
</tr>
<tr>
<td></td>
<td>Methylcholanthrene</td>
<td>1.47 ± 0.05^a</td>
<td>0.58 ± 0.02^a</td>
</tr>
<tr>
<td>Female rats</td>
<td>0.9% NaCl solution</td>
<td>0.66 ± 0.17</td>
<td>0.67 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>Phenobarbital</td>
<td>15.44 ± 4.20^a</td>
<td>0.64 ± 0.05</td>
</tr>
<tr>
<td>Female mice</td>
<td>0.9% NaCl solution</td>
<td>9.69 ± 0.72</td>
<td>0.68 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>Phenobarbital</td>
<td>65.72 ± 12.29^a</td>
<td>0.48 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>Methylcholanthrene</td>
<td>9.45 ± 0.20</td>
<td>0.52 ± 0.05</td>
</tr>
</tbody>
</table>

^a Statistically different from values obtained when microsomes from the respective 0.9% NaCl solution-treated controls were used ($p < 0.05$).

### Table 2

**Depression of rat hepatic microsomal metabolism of cyclophosphamide by pretreatment with thioacetamide, morphine, or cobalt chloride**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$\nu$ (µmoles/g/hr)</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9% NaCl solution</td>
<td>1.85 ± 0.10</td>
<td>12</td>
</tr>
<tr>
<td>Thioacetamide</td>
<td>0.22 ± 0.01</td>
<td>48</td>
</tr>
<tr>
<td>Morphine</td>
<td>0.89 ± 0.13</td>
<td>8</td>
</tr>
<tr>
<td>Cobalt chloride</td>
<td>0.15 ± 0.06</td>
<td>8</td>
</tr>
</tbody>
</table>

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The antineoplastic activity of cyclophosphamide and/or its metabolites was evaluated with the use of the Walker 256 carcinosarcoma grown i.m. Injection of 1 million tumor cells obtained from ascites fluid into the hindleg muscle of rats resulted in the growth of a solid tumor. This tumor grew with an approximate $T_D$ (doubling time) of 1.05 days for the 1st 7 days and an approximate $T_D$ of 19.5 days thereafter (Chart 3). The $T_D$ of the first part of the growth curve is somewhat difficult to estimate and, in fact, the experimental values obtained on Days 2, 3, 4, and 5 after the introduction of the tumor cells do not fall on the line drawn in Chart 3. If the line is drawn through the points, a theoretical value of about 2 to 3 g is obtained for the weight of the tumor at zero time, i.e., at the time the tumor was injected. This, quite obviously, cannot be the case, since the tumor cells were injected in suspension in a total volume of only 0.1 ml. A more likely explanation is that the introduction of the tumor cells initiates an inflammatory response and that this subsides with time. Because the estimate of tumor weight was obtained by subtracting the weight of the control leg from the tumor-bearing leg, any weight due to inflammation would also be interpreted as tumor weight. A more precise way to determine actual tumor weight would be to dissect out the tumor and weigh it directly. This approach was not used because of the difficulty in determining accurately and easily what is tumor tissue and what is normal tissue with this system. An additional problem in estimating the $T_D$ of the initial growth phase is the duration of the inflammatory response, if such exists. Because the line depicting the initial growth phase in Chart 3 falls on the experimental values without extracting a significant amount of the metabolites, possibly except for nor-HN2 (16).

No alkylating activity was found in blood 24 hr after the administration of cyclophosphamide, regardless of how the animals were pretreated. In male rats, peak blood levels of alkylating activity were reached at about 1 hr after injection of cyclophosphamide. Relative to control male rats, blood levels of alkylating activity were markedly elevated at early time periods in male rats pretreated with phenobarbital, with maximal levels being reached between 15 and 30 min after injection of cyclophosphamide. In these animals, alkylating activity appeared to disappear from blood at a rate much faster than in controls, an observation previously reported in man (22) as well as in rats and mice (2, 22, 23). Thus, blood levels of alkylating activity were much lower in phenobarbital-pretreated male rats 2 hr after the injection of cyclophosphamide. Relative to control male rats, blood levels of alkylating activity were markedly lower at all time points measured up to 2 hr following the injection of cyclophosphamide. By 3 hr, however, essentially no differences remained. In all cases, qualitatively similar results were obtained when blood was first extracted with methylene chloride before analysis for alkylating activity, indicating that the results reflect differences in metabolite rather than cyclophosphamide levels. Thus, the differences in rates of cyclophosphamide metabolism observed in vitro were qualitatively paralleled in vivo, suggesting that most, if not all, of cyclophosphamide metabolism to alkylating metabolites occurs in the hepatic endoplasmic reticulum.
obtained on Days 6 and 7 and extrapolates to 0.1 g at Day 0, it was felt that this line most accurately reflects tumor weight and that the assumed inflammatory response has subsided by Day 6. A quantitatively and qualitatively similar growth curve was obtained when female rats were used as the host animals (data not presented).

Kato et al. (19, 20) have shown that rats bearing the Walker 256 carcinosarcoma metabolize many drugs, including cyclophosphamide, at a depressed rate. The current investigations, in which the rate of cyclophosphamide metabolism in vitro was determined as a function of the weight of the tumor in the rat from which hepatic microsomes were obtained, showed that the introduction of 1 million tumor cells had little effect for 3 days but that, thereafter, hepatic microsomal mixed-function oxidase activity declined precipitously reaching a minimum (20% of control) 8 days after the introduction of the tumor cells (Chart 4). The fall in drug-metabolizing activity ceases at about the same time that the tumor cells enter into the 2nd growth phase. Little or no further decrease in activity was observed 11 days after tumor cells were injected. In these experiments, male rats were used. Essentially the same results were obtained when female rats were used, although the minimum was about 40% of control in this sex (data not presented). *In vivo* cyclophosphamide metabolism was also depressed when evaluated 9 days after introduction of the tumor (Charts 1 and 2).

High doses of cyclophosphamide have been reported to depress hepatic microsomal mixed-function oxidase activity (12, 30). However, cyclophosphamide, at an i.p. dose of 25 mg/kg, once daily for 4 days, did not depress this activity as evaluated by ethylmorphine *N*-demethylation in our laboratory (data not presented). Furthermore, even when cyclophosphamide was given at a high dosage level, 150 mg/kg, hepatic microsomal mixed-function oxidase activity was not measurably depressed until 5 days after injection of the drug (30).

We estimated the antineoplastic activity of cyclophosphamide by injecting 1 million Walker 256 carcinosarcoma cells, ascites form, into the hindleg muscles of rats and, 24 hr later, injecting cyclophosphamide at various dose levels. Seven days after the introduction of tumor cells, animals were sacrificed and tumor weight was determined. Dose-response curves and ED₅₀’s were then plotted and estimated, respectively. Cyclophosphamide was injected into the animals 24 hr after introduction of tumor cells because, at this time, presence of the tumor has little effect on hepatic microsomal mixed-function oxidase activity (Chart 4). Since all of the drug to be metabolized is essentially metabolized in less than 6 hr, the total amount of metabolism accurately reflects the total enzyme activity present 24 hr after introduction of the tumor cells and is not complicated by the decrease in metabolism resulting from tumor growth. For this reason, too, cyclophosphamide was injected only once. Animals pretreated with phenobarbital, 3-methylcholanthrene, or cobalt chloride, were injected with these compounds at dosage levels and on schedules such that a maximal effect,
either increase or decrease in metabolism, was achieved at the time cyclophosphamide was injected. Tumor weight was determined 7 days after introduction of the tumor cells because, at this time, the 1st part of the growth phase is nearing its end.

With the use of the system described, cyclophosphamide inhibited tumor growth in untreated male rats with an ED50 of 0.69 mg/kg (Table 3). Although phenobarbital pretreatment stimulates the rate of cyclophosphamide activation, no change relative to control in ED50 or in slope of the dose-response curve was observed (Table 3). Relative to male rats, female rats metabolize cyclophosphamide at a much slower rate, and yet there are no differences in ED50’s or in the slopes of the dose-response curves (Table 3). In contrast, following 3-methylcholanthrene pretreatment, the rate of metabolism is depressed and the ED50 is increased (Table 3). Cobalt chloride pretreatment depresses the rate of cyclophosphamide activation, but the ED50 is not increased. This latter experiment is more difficult to interpret, because the slope of the dose-response curve is also somewhat altered (Table 3).

As is the case with virtually all antineoplastic agents, one of the major undesired effects of cyclophosphamide is leukopenia. Thus, leukocyte counts in peripheral blood were made at various times after the injection of a given dose of cyclophosphamide as a quantitative estimate of toxicity. Although, with the exception of 3-methylcholanthrene pretreatment, an increase or decrease in the rate of cyclophosphamide activation did not alter the desired therapeutic response, alterations in the undesired toxic response might be produced, thereby increasing or decreasing the therapeutic index. As before, animals pretreated with phenobarbital, 3-methylcholanthrene, or cobalt chloride were injected with these compounds, with dosage levels and schedules such that a maximal effect, either an increase or a decrease in metabolism, was achieved at the time of cyclophosphamide injection.

Preliminary experiments (data not presented) established that leukopenia due to cyclophosphamide and/or its metabolites is log dose dependent, with a 50% depression in leukocyte counts achieved at a dose of 13 mg/kg. Following the administration of 1 dose of cyclophosphamide, 50 mg/kg, to otherwise untreated male rats, the leukocyte count decreased steadily, reaching a nadir 4 days postinjection (Chart 5). At this time, the leukocyte count was about 18% of the initial count. Nine days postinjection, the leukocyte count had returned to about 65% of control. These findings are substantially in agreement with previous reports (10, 32). Essentially the same response was obtained if cobalt chloride-pretreated male rats were used. In 3-methylcholanthrene-pretreated male rats and in female rats, the rate of leukocyte depression was similar to that observed in male rats, but the magnitude of depression was somewhat less, and recovery at 9 days was smaller and greater, respectively. When phenobarbital-pretreated male rats were used, the rate and magnitude of leukocyte depression due to cyclophosphamide were greater relative to that observed in male rats pretreated with 0.9% NaCl solution, and reached a nadir 3 days after the injection of cyclophosphamide. The rate of recovery was similar to that observed in untreated male rats. Phenobarbital, 3-methylcholanthrene, and cobalt chloride did not alter the leukocyte count over the 9-day period, when administered by themselves. Thus, phenobarbital pretreatment, which stimulates the rate of cyclophosphamide activation, would seem to hasten the onset and increase the magnitude of toxicity. In animals in which the rate of cyclophosphamide activation is depressed relative to untreated male rats, i.e., in female rats and in cobalt chloride- or 3-methylcholanthrene-pretreated male rats, there appear to be only minor differences in the rate of appearance and magnitude of toxicity, although there are some differences in recovery rates. Differential counts of granulocytes (neutrophils) and agranulocytes (lymphocytes) showed essentially the same patterns, although there was great variability in the neutrophil counts (data not presented).

**DISCUSSION**

It has been well established that cyclophosphamide itself is virtually without alkylating and cytotoxic activity relative to its metabolite(s) and that the activation of cyclophosphamide in vitro is catalyzed by the same hepatic microsomal mixed-function oxidases that are operative in the oxidative metabolism of many other drugs (2, 4, 5, 7, 8, 26). The current experiments demonstrated that the rate of cyclophosphamide activation could be stimulated or depressed...
by pretreatment with various drugs. In vivo, an increased activation rate was accompanied by an apparent increased disappearance rate of alkylating activity from blood, and vice versa. However, assuming, as might be expected, that blood levels of alkylating activity following i.p. cyclophosphamide administration are largely governed by 1st-order activation and 1st-order elimination kinetics, the altered blood levels of alkylating activity observed after pretreatment with stimulators and depressors of cyclophosphamide activation can be accounted for solely by altered cyclophosphamide activation rates, precluding an effect of these compounds on those factors, e.g., blood flow, enzyme concentration, that contribute to the kinetic constants describing rates of excretion, inactivation and distribution (3, 15). Thus, relative to the response obtained in control male rats, stimulation of activation, e.g., with phenobarbital pretreatment, should and does result in an increased concentration of active drug at early times but in a lowered concentration of drug at early times but in an elevated concentration of active drug at later times.

The therapeutic response to a drug depends not only upon the intrinsic properties of the drug that enable it to interact with a given receptor site but also upon those physicochemical features of the drug that dictate its absorption, distribution, excretion, and biotransformation.

Drugs can be divided into 2 groups: those that produce "graded," reversible responses, and those that produce "all-or-none," irreversible effects (1). Increase and decreases in biotransformation rates are known to produce quantitative changes in the intensity and duration of action of drugs that effect graded responses (21). The response produced by drugs that produce an all-or-none effect, e.g., tumor cell kill, depends on both concentration of and contact time with the drug. Changes in biotransformation rates of these drugs when the parent compound is the active species would also be expected to produce quantitative changes in the therapeutic response. However, in those cases in which the metabolite rather than the parent compound is the active species, little or no quantitative change in therapeutic response might be expected, provided that the metabolite is distributed in total body water, that tissue concentration of the metabolite is governed by 1st-order activation and 1st-order elimination kinetics, and that the active metabolite but not the parent compound is readily excreted.

Expectations of the type described were observed in the current experiments. A stimulated rate of cyclophosphamide activation in rats pretreated with phenobarbital and depressed rates of cyclophosphamide activation in female rats or in rats pretreated with cobalt chloride did not result in an altered therapeutic response, i.e., ED50. Similarly, significant alterations in the undesired toxic response, leukopenia, were not observed, as would be expected if undesired and desired toxicities result from the same action of the drug. Thus, the therapeutic index of cyclophosphamide is not altered by increasing or decreasing its rate of activation.

Depressed rates of cyclophosphamide activation following 3-methylcholanthrene pretreatment did not result in the expected therapeutic or toxic response, i.e., ED50. Similarly, significant alterations in the undesired toxic response, leukopenia, were not observed, as would be expected if undesired and desired toxicities result from the same action of the drug. Thus, the therapeutic index of cyclophosphamide is not altered by increasing or decreasing its rate of activation.

The data described herein provide experimental evidence for the theoretical prediction that the therapeutic efficacy of cyclophosphamide cannot readily be altered by pretreating animals with drugs that stimulate or depress the rate of cyclophosphamide activation and demonstrate the futility of trying to improve the therapeutic efficacy of cyclophosphamide by pretreatment with drugs that alter its rate of activation. In general, similar predictions can be made for any compound that is not itself biologically active and that produces an all-or-none response after conversion to an active metabolite.

Competitive and noncompetitive inhibitors of cyclophosphamide activation should also fail to alter the therapeutic efficacy of cyclophosphamide. Experiments designed to test
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the validity of this prediction are currently in progress and will be the substance of a future communication.

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