Acute Lethality for Mice Following Administration of Cyclophosphamide with Barbiturates (37241)

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Cyclophosphamide (NSC 26271) is a potent antineoplastic agent of the nitrogen mustard family. It is converted by enzymes present in hepatic microsomes to an active metabolite possessing cytotoxic properties (1, 3, 5, 8, 9). Because of this activation step, the effects on toxicity and therapeutic activity of cyclophosphamide by agents that modify microsomal enzyme activity have been studied. Inhibitors of microsomal enzyme activity such as SKF 525A and chloramphenicol were found to decrease the alkylating activity and toxicity associated with cyclophosphamide administration (1, 4, 8). Stimulation of microsomal enzyme activity by chlordane resulted in an increased cyclophosphamide-associated lethality or a reduced mean time of onset of death or both (3).

Previous studies involving interactions between barbiturates and cyclophosphamide have focused on the effects of prior drug treatment on the degree of leukopenic (9), alkylating (5), cytotoxic (1, 5), or lethal (3, 4, 8) activity of cyclophosphamide. For example, mice pretreated with phenobarbital, so as to induce microsomal enzyme activity, experienced an increase in cyclophosphamide lethality during the early part of the observation period (8). Such results indicate that the lethal and cytotoxic effects of cyclophosphamide are associated with metabolites rather than the parent compound (9). Gibson and Becker (6), however, found that cyclophosphamide teratogenicity and embryo toxicity were increased in pregnant mice pretreated with SKF 525A and decreased in pregnant mice pretreated with phenobarbital. Moreover, increased cyclophosphamide toxicity was observed in infant mice whose ability to metabolize the drug was less than that of adult mice (13); therefore, cyclophosphamide may manifest toxic activity without undergoing prior metabolism.

The present report describes an *in vivo* interaction between barbiturates and cyclophosphamide resulting in a rapid onset of death. The results of this study are of particular importance because barbiturates are recommended to alleviate gastrointestinal and central nervous system disturbances elicited by certain alkylating agents. Additionally, with the advent of protective environments and supportive therapy, *i.e.*, platelet and leukocyte transfusion, large doses of anticancer drugs are being considered.

Materials and Methods. BALB/c mice weighing 20–23 g were used. They were allowed to adjust to their new environment for at least 1 wk prior to experimentation and were fed a commercial food preparation and water *ad libitum*.

Cyclophosphamide (Mead Johnson Laboratories, Evansville, IN), sodium hexobarbital (Sterling-Winthrop Laboratories, New York, NY), sodium pentobarbital (Diamond Laboratories, Des Moines, IA), and sodium phenobarbital (Sterling-Winthrop) were dissolved in physiologic saline. Doses of the barbiturates were expressed in milligrams of the sodium salt. Except for doses of 750 and 1000 mg cyclophosphamide/kg, which were administered in 0.015 and 0.02 ml/g mouse, respectively, all drug solutions were prepared such that the required amount could be administered in 0.01 ml/g mouse. All injections were by the intraperitoneal (ip) route unless otherwise specified. Mice receiving two drugs received each solution separately unless otherwise indicated. In experiments in which test

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mice received more than 0.5 ml of drug solutions, control mice were administered 0.15 *M* NaCl so that animals receiving one or no drug received the same volume of fluid as the test animals.

Pretreatment of mice with phenobarbital consisted of ip injection of 65 mg/kg given once daily for 4 days; mice were challenged 1 day after the last injection.

Probit analyses of data leading to LD_{50} determinations, tests for parallelism and calculation of potency ratios were done by the method of Litchfield and Wilcoxon (12). The acceptable level of significance was $p < 0.05$.

Assay for aminopyrine metabolism was done by the method of Gram, Wilson and Fouts (7) using the 9000*g* mouse liver supernatant fraction. Assay for pentobarbital present in the brains of mice was done by the method of Brodie *et al.* (2) using sodium pentobarbital as the standard.

In vivo metabolism of barbiturates was estimated using sleeping times after drug administration. Pentobarbital (45 mg/kg), hexobarbital (80 mg/kg) or phenobarbital (80 or 100 mg/kg) was injected ip and the time interval in minutes between loss of righting reflex and recovery of reflex was recorded. Recovery was defined as the ability of the mice to right themselves twice within a 10 sec period.

Results. The simultaneous administration of pentobarbital and cyclophosphamide resulted in a rapid onset of death (Fig. 1). Although 1000 mg cyclophosphamide/kg failed to kill any mice within the first hour after injection, cyclophosphamide given in combination with 45 mg pentobarbital/kg resulted in an LD_{50} with respect to cyclophosphamide of 425 mg/kg. Thus, despite our inability to determine the LD_{50} of cyclophosphamide 1 hr after challenge because the solubility of the drug was limiting, the lethality resulting from these combinations with pentobarbital represented more than a 2-fold potentiation of cyclophosphamide toxicity. By 24 hr after injection, the potentiation of lethality with respect to cyclophosphamide, administered with 45 mg pentobarbital/kg, dissipated.

Unlike cyclophosphamide, death due to pentobarbital occurred within 1 hr of injection. The LD_{50} of pentobarbital determined 1 hr after its administration was 131 mg/kg (95% confidence interval = 126 to 140). The steep dose–response curve of pentobarbital (slope = 1.26; 95% confidence interval = 1.15 to 1.37) was potentiated in a parallel manner by simultaneous administration of 375 mg cyclophosphamide/kg (slope = 1.21; 95% confidence interval = 1.08 to 1.33). The reduction in LD_{50} with respect to pentobarbital, from 131 mg/kg to 51 mg/kg (95% confidence interval = 49 to 53), represents a potency ratio of 2.6-fold. This value was the same whether determined 1 or 24 hr after challenge.

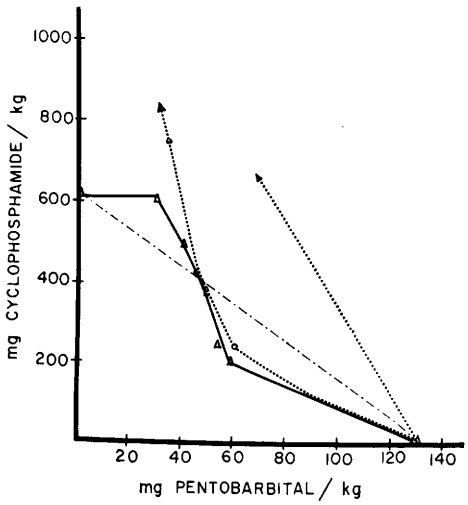

FIG. 1. Synergistic toxicity for mice of combinations of pentobarbital with cyclophosphamide. The solid line connects LD_{50} values after 24 hr for various combinations; (——) the responses expected for additive effects; (- - -) connects LD_{50} values after 1 hr for various combinations; (• • • •) projects the responses expected for additive effects.

The enhanced lethality involving cyclophosphamide was not restricted to pentobarbital. Simultaneous administration of cyclophosphamide with nonlethal doses of hexobarbital resulted in marked lethality for mice within 1 hr following challenge. For example, a dose of 500 mg cyclophosphamide/kg in combination with 100 mg hexobarbital/kg killed 50% of 10 treated mice within 1 hr and 80% within 24 hr although 200 mg hexobarbital/kg alone did not kill any mice within 24 hr. Cyclophosphamide alone (500 mg/

kg) did not kill any animals within 1 hr and only 10% within 24 hr. A dose of 500 mg cyclophosphamide/kg in combination with 60 mg hexobarbital/kg did not kill any mice within 1 hr but 30% of 20 treated animals were dead within 24 hr.

Nonlethal and minimally lethal doses of phenobarbital elicited substantial mortality within 1 hr after injection with cyclophosphamide. For example, a dose of 500 mg cyclophosphamide/kg in combination with 130 mg phenobarbital/kg killed 15% of 20 treated mice within 1 hr and 70% within 24 hr although 195 mg phenobarbital/kg did not kill any mice within 1 hr and only 10% within 24 hr. A dose of 750 mg cyclophosphamide/kg in combination with 130 mg phenobarbital/kg killed 45% of 20 treated mice within 1 hr and 85% within 24 hr although 750 mg cyclophosphamide/kg did not kill any mice within 1 hr. This dose of cyclophosphamide however killed 90% of 20 treated animals within 24 hr.

When 375 mg cyclophosphamide/kg was given at various time intervals with respect to 45 mg pentobarbital/kg, simultaneous administration of the two agents was found to result in maximal lethality. When this dose of cyclophosphamide was given to groups of 10 mice 6, 3 or 1 hr before pentobarbital or when cyclophosphamide was given 1 hr after pentobarbital, no death occurred. Mortality was measured, as above, 1 and 24 hr after administration of the last drug. Various routes of injection were investigated. The rate and magnitude of the lethality were not altered by iv, ip, or various combinations of the two routes. Additionally, the synergy occurred when both drugs were administered orally. The lethal response also was elicited when the drugs were mixed *in vitro* prior to administration.

Barbiturate-induced sleep was prolonged in mice given cyclophosphamide concurrently. For example, the duration of pentobarbital-induced (45 mg/kg) sleep was 46.6 ± 7.0 min (mean ± SE) whereas that for mice simultaneously given 150 mg cyclophosphamide/kg was 99.8 ± 9.0 min. Two of the 10 mice receiving 45 mg pentobarbital/kg and 150 mg cyclophosphamide/kg died within 1 hr. Similarly, the mean duration of hexobarbital-induced (80 mg/kg) sleep was 28.3 ± 4.9 min; whereas that for mice simultaneously given 375 mg cyclophosphamide/kg was 147.4 ± 8.4 min. None of the hexobarbital-treated animals died within 1 hr. Of 10 mice receiving 100 mg phenobarbital/kg, only three lost the righting reflex (time for onset of sleep = 42.6 min; duration of sleep = 15.2 min). All 10 mice receiving 100 mg phenobarbital/kg and 500 mg cyclophosphamide/kg lost the righting reflex (time for onset of sleep 12.9 min; duration of sleep > 117.1 min). Of 10 mice receiving 80 mg phenobarbital/kg, none lost the righting reflex whereas all 10 mice receiving this dose of phenobarbital in combination with 500 mg cyclophosphamide/kg did (time for onset of sleep = 13.2 min; duration of sleep > 163.6 min). Significantly, 80% of the animals receiving phenobarbital and cyclophosphamide died within 24 hr.

Mice pretreated with multiple injections of phenobarbital were subsequently challenged with either 500 mg cyclophosphamide/kg alone or in combination with 45 mg pentobarbital/kg (Table I). Pretreated mice were significantly ($p < 0.025$) protected from the combination of 500 mg cyclophosphamide/kg with 45 mg pentobarbital/kg, as determined 1 hr and 24 hr after challenge, when compared to control mice. Also the duration of hexobarbital-induced sleep was shorter with phenobarbital-treated mice surviving the simultaneous challenge with cyclophosphamide and hexobarbital than normal mice receiving the same challenge. Despite the protection against the combination of cyclophosphamide and pentobarbital, phenobarbital-pretreated mice were rendered hyperreactive to cyclophosphamide alone, as judged by the significant ($p < 0.05$) increase in lethality suffered by these mice 24 hr after challenge.

Liver homogenates were prepared following challenge with cyclophosphamide. The 9000*g* liver supernatant fractions were compared to preparations derived from mice not receiving cyclophosphamide for their ability to metabolize aminopyrine (Table II). Analysis of the K_m and V_{max} of each preparation with regard to aminopyrine metabolized in 30 min revealed no remarkable differences between liver supernatant fractions derived

TABLE I. Effect of Phenobarbital Pretreatment of Cyclophosphamide–Pentobarbital Lethality.

Group	Cyclophosphamide (mg/kg)	Pentobarbital (mg/kg)	No. mice	Cumulative % dead by 1 hr	Cumulative % dead by 24 hr
		Normal			
1	500	—	30	0	10
2	500	45	30	50	60
		Pretreated[a]			
3	500	—	30	0	37[b]
4	500	45	30	17[c]	27[c]

[a] Pretreatment consisted of 65 mg phenobarbital/kg once daily for 4 days (ip). Mice were challenged 1 day after the last injection.
[b] $p < .05$ (Group 1 vs Group 3; 24 hr).
[c] $p < .025$ (Group 2 vs Group 4).

from cyclophosphamide-treated and normal mice.

Mice were given pentobarbital with and without 500 mg cyclophosphamide/kg 15 min prior to sacrifice and removal of their brains; the relative amounts of pentobarbital present in the brains of each treatment group were compared (Table III). It should be noted that 15 min was the time when deaths began to occur. Although cyclophosphamide was observed to cause an increase in the amount of pentobarbital recovered following injection of 45 mg pentobarbital/kg ($p < 0.05$), the level attained was less than that found after administration of a nonlethal dose of 90 mg pentobarbital/kg.

Discussion. Our data indicate that the synergistic action of barbiturates and cyclophosphamide is not mediated through a cyclophosphamide metabolite. This is predicated on the very early (< 15 min) deaths and even earlier cholinergic signs, *i.e.*, salivation, respiratory distress and a dart-like appearance at death. Additionally, phenobarbital pretreatment, which is known to enhance the alkylating type toxicity of cyclophosphamide, does not enhance the synergistic response. Quite the opposite occurred, the phenobarbital-pretreated mice were protected from the enhanced lethality. This is probably due to an increased metabolism of pentobarbital. Additionally, the microsomal enzymes responsible for metabolizing the Type I substrate aminopyrine are not remarkably affected at the time when the lethality occurs.

TABLE II. Effect of Cyclophosphamide on Microsomal Enzyme Type I Activity.[a]

Treatment (mg/kg ip)	K_m[b]	V_{max}[c]
Control	0.748 ± 0.100 (7)[d]	150 ± 2.6 (7)[d]
Cyclophosphamide		
250	0.829 ± 0.152 (3)	141 ± 5.1 (3)
500	0.845 ± 0.105 (4)	145 ± 3.9 (4)
750	0.832 ± 0.287 (2)	140 ± 7.7 (2)

[a] Cyclophosphamide administered ip 1 hr prior to enzyme assay.
[b] K_m (mM).
[c] μg HCHO/g liver/30 min.
[d] Values in parentheses represent the number of experiments. Each K_m and V_{max} was determined from at least 18 points on the curve.

We do not know whether the barbiturate is enhancing the lethality of the alkylating agent or vise versa or that both drugs are enhancing the toxicity of each other. The rapid onset of death and the parallelism of the barbiturate dose–response curve in the presence and absence of a constant dose of cyclophosphamide indicate that the barbiturate toxicity is being enhanced. However, the level of barbiturate in the brains of mice given cyclophosphamide and pentobarbital is not sufficiently elevated to corroborate this proposition. On the other hand, it is known that alkylating agents, particularly nitrogen mustard, possess cholinergic properties and central nervous system stimulating activity (11) and that the mice dying after administration of a barbiturate with cyclophospha-

TABLE III. Effect of Cyclophosphamide on Pentobarbital Levels in Mouse Brain.

Treatment[a] (mg/kg)		Pentobarbital recovered (μg/g brain)[b]
Pentobarbital	Cyclophosphamide	
45	—	97.9 (3)
45	500	108.1 (4)
90	—	138.4 (2)

[a] All drugs were given ip 15 min before sacrifice.

[b] Extracted and assayed according to the method of Brodie *et al.* (2) Each value represents the mean for the number of experiments shown in parentheses. The standard error of the mean was less than 6% in each instance. Each experiment utilized 3 BALB/c mouse brains.

mide show cholinergic signs. The possibility exists that an increased amount of alkylating agent crosses the blood–brain barrier in the presence of the barbiturate causing the lethality. Alternatively, one drug may increase the susceptibility of the central nervous system to the toxic action of the other agent. Another mechanism may be the release of a vasoactive substance as is suggested by Herman *et al.* (10), who recently reported that cyclophosphamide administered to barbiturate-anesthesized rhesus monkeys produced an immediate dose-dependent hypotension, bradycardia, and depressed EEG activity. This was associated with elevation of blood histamine.

It is not unusual for patients to receive barbiturates while undergoing antitumor therapy; indeed with certain alkylating agents, barbiturate is recommended to alleviate side effects. Until the nature of this drug interaction is further investigated, the clinician should be cautious in using these drugs together.

Summary. The simultaneous administration of cyclophosphamide and pentobarbital resulted in a rapid onset of death for BALB/c mice. When the drugs were given 1 hr apart, in either order, the mice survived. Pentobarbital lethality was potentiated in a parallel manner by 375 mg cyclophosphamide/kg. Hexobarbital and phenobarbital successfully substituted for pentobarbital in eliciting enhanced lethality when administered with cyclophosphamide. Cyclophosphamide increased the duration of sedation elicited by each barbiturate. Pretreatment of mice with multiple phenobarbital injections diminished the lethal action of subsequent challenge with cyclophosphamide and pentobarbital. However, mice pretreated with phenobarbital were hyperreactive to cyclophosphamide alone. The 9000*g* liver supernatant fraction derived from cyclophosphamide-treated mice was about equal in metabolizing aminopyrine as was the 9000 g liver supernatant fraction derived from normal mice. Cyclophosphamide had a minor effect on the amount of pentobarbital recovered from mouse brain tissue 15 min after injection, compared to mice receiving pentobarbital alone.

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