A Correlation Between Cyclophosphamide Induced Leukopenia in Mice and the Presence of Alkylating Metabolites (36157)

FORREST D. HAYES, ROBERT D. SHORT, AND JAMES E. GIBSON
(Introduced by D. A. Reinke)
Department of Pharmacology, Michigan State University, East Lansing, Michigan 48823

Cyclophosphamide (2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino] tetrahydro-2-oxide, Cytoxan, Endoxan, NSC-26271) is a clinically useful antineoplastic agent of the alkylating type. The molecule was designed to require bioactivation for the production of alkylating metabolites (1). Cyclophosphamide activation has been shown to occur primarily in the mixed function oxidase system of the liver microsomal fraction (2). The cytotoxic effect of cyclophosphamide, in mammalian tissue cultures, has been associated with metabolites rather than the parent compound (3).

Rats pretreated with either phenobarbital (4) or cholordane (5), to induce microsomal mixed function oxidase enzyme activity, had an increased *in vivo* (4) and *in vitro* (5) ability to convert the parent compound to alkylating metabolites. Cyclophosphamide activation, on the other hand, was reduced in rats pretreated with either SKF 525-A (β-diethylaminoethyl-diphenylpropyl acetate) (2) or chloramphenicol (6), which were both inhibitors of the microsomal enzyme system. An increase in cyclophosphamide lethality and reduced mean time to death was observed in rats pretreated with either phenobarbital (7) or chlordane (5). Chloramphenicol pretreatment, on the other hand, reduced cyclophosphamide lethality and increased the mean time to death (6). The lethality of cyclophosphamide in mice, during a 28-day observation period, was reduced by SKF 525-A pretreatment and not altered by phenobarbital pretreatment (8). There was, however, an increase in cyclophosphamide lethality in phenobarbital-pretreated mice during the early part of the observation period. These results suggest that both the lethal and cytotoxic effects of cyclophosphamide were associated with metabolites rather than the parent compound. Cyclophosphamide teratogenicity and embryotoxicity, on the other hand, were increased and decreased, respectively, by pretreatment of pregnant Swiss-Webster mice with SKF 525-A and phenobarbital (9). Furthermore, infant mice, which have practically no ability to activate cyclophosphamide, were more sensitive to cyclophosphamide toxicity than adults (10). Cyclophosphamide, therefore, may produce toxic actions by nonalkylating as well as alkylating mechanisms.

The leukopenic action of alkylating agents is well known and initial experiments indicated that cyclophosphamide produced a dose-related decrease in the total white blood cell count in Swiss-Webster mice. Since perinatal mice of this strain appear sensitive to a nonalkylating form of cyclophosphamide, experiments were conducted to determine adult sensitivity to cyclophosphamide. The role of the parent compound and its metabolites in the production of adult toxicity was evaluated by determining the effect of phenobarbital and SKF 525-A pretreatment on the leukopenic action of cyclophosphamide in mice.

Methods. Mature female Swiss-Webster mice (Spartan Farms, Haslett, MI) were housed in groups of five in stainless-steel wire bottom cages at 70–75°F. The mice were fed Wayne Lab-Blox (Allied Mills, Inc., Chicago, IL) and tap water *ad libitum*. The light–dark cycle was 12 hr in duration and began with light on at 8 a.m.

Peripheral circulating blood was obtained

at 8 a.m. once before and at various times after drug treatment. Samples were obtained at the same time each day to minimize diurnal variation in mouse leukocyte counts (11). Blood was collected by excision of a posterior segment of tail that had been rubbed to stimulate blood flow and decrease venous stagnation. All samples were collected in disposable white cell blood diluting pipettes (Unopette, Becton, Dickinson and Co., Rutherford, NJ). Total white cell counts were made using a hemacytometer (Spencer Improved Neubauer type). Differential leukocyte counts were determined from a dried blood smear stained with Wright's stain and examined microscopically using an oil immersion lens.

Cyclophosphamide was administered intraperitoneally in a freshly prepared aqueous solution at a single dose of 0, 75, 150, or 300 mg/kg (0.01 ml/g of body wt) to determine the dose and temporal aspects of the drug-induced leukopenia. Cyclophosphamide (75 mg/kg) was also administered to groups of mice pretreated with either phenobarbital (50 mg/kg ip twice a day for 3 days) or SKF 525-A (40 mg/kg ip 1 hr prior to cyclophosphamide administration). Control animals received either distilled water, phenobarbital, or SKF 525-A. A minimum of 5 animals received each treatment.

The effect of phenobarbital and SKF 525-A on the activation of cyclophosphamide by female mouse liver was demonstrated *in vitro*. Nonpretreated mice or mice pretreated with either phenobarbital (see above) or SKF 525-A (32 mg/kg 1 hr prior to sacrifice) were sacrificed by cervical dislocation; and their livers were perfused with 1.15% KCl in 0.5 *M* tris(hydroxymethyl)aminomethane (Tris, Sigma, St. Louis, MO), pH 7.4 (KCl–Tris), prior to removal. The tissues were maintained in an ice bath for up to 1 hr prior to being weighed and homogenized in 2 ml of KCl-Tris/g of tissue. The tissues were homogenized in a loosely fitting, motor driven, Teflon–glass Potter-Elvehjem homogenizer.

The tissue fraction for incubation was obtained by centrifuging the homogenate at 9000*g* for 30 min in an International centrifuge Model B-20.

The *in vitro* system contained cyclophosphamide, the 9000*g* supernatant, glucose-6-phosphate (Sigma), nicotinamide adenine dinucleotide phosphate (Sigma), magnesium sulfate ($MgSO_4$), and 0.1 *M* Tris (pH 7.4). The components were added in a volume of 2.5 ml to a 50 ml beaker containing a marble; and incubations were conducted at 37° in room air using a Dubnoff metabolic shaking apparatus. The reaction was terminated by the addition of 0.5 ml of 95% ethanol. Alkylating metabolites in the supernatant were quantified using nitrobenzyl pyridine (NBP) (9, 12) and an acid hydrolyzed cyclophosphamide standard. Protein determinations were made on the 9000*g* supernatant by the method of Lowry *et al.* (13) and crystalline bovine serum (Armour) served as a standard.

The *in vitro* system was optimized and the kinetics of activation were determined (10). The K_m was estimated to be 0.92 m*M* using a Lineweaver-Burk plot and the reaction was linear for at least 10 min. The 9000*g* supernatant of livers from control and phenobarbital- or SKF 525-A-pretreated mice were incubated for 5 min in the optimized system with a cyclophosphamide concentration of 12.8 m*M*.

The test of significance was calculated by Students' *t* test and the level of significance was chosen as $p < .05$ (14).

Results. Table I shows that phenobarbital pretreatment significantly increased the *in vi-*

TABLE I. Effect of Phenobarbital and SKF 525-A on *in Vitro* Cyclophosphamide Activation and Liver/Body Weight Ratio of Female Mice.

Pretreatment	Alk Act [a] (mμmoles /mg of protein)	g of liver / 100 g of body
Control	9.0 ± 0.9 (6)[b]	4.5 ± 0.2 (6)
Phenobarbital	21.0 ± 2.3 (5)[c]	5.6 ± 0.2 (6)[c]
SKF 525-A	3.2 ± 0.6 (6)[c]	4.3 ± 0.1 (6)

[a] Five minute incubation (Alk Act = alkylating activity).

[b] Mean ± SE (no. of observations).

[c] Significantly different from control ($p < .05$).

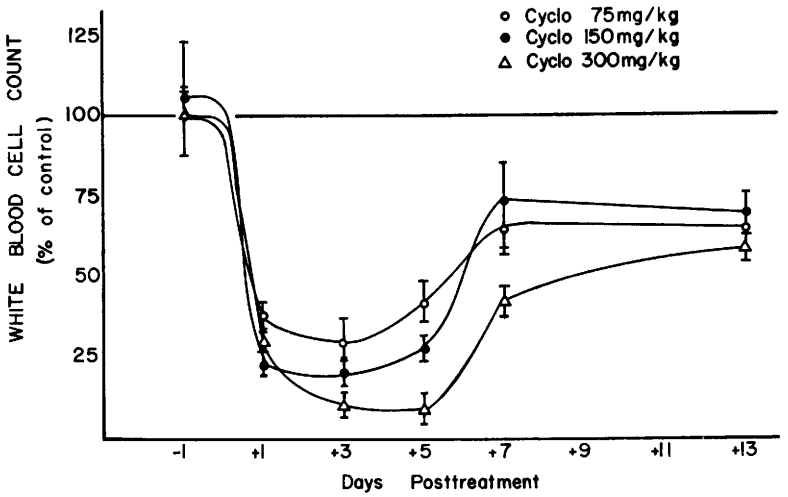

FIG. 1. White blood cell counts in cyclophosphamide-treated mice relative to simultaneously determined control values as a function of days after drug treatment: The values plotted represent the mean percent of control value ± SE for 5 determinations. The control value was determined at each of the indicated times after treatment. The average control total white blood cell count ranged from 7500 to 12,500 cells/mm^3.

tro ability of livers from female mice to convert cyclophosphamide to NBP alkylating metabolites. SKF 525-A pretreatment, on the other hand, significantly reduced the ability of the liver to activate cyclophosphamide. The liver/body weight ratio was significantly increased by phenobarbital pretreatment and unchanged by SKF 525-A pretreatment. This data confirmed *in vivo* observations in mice (9) and indicated that the pretreatment drugs produced their observed effects on the activating system by an action on the liver.

The effect of various cyclophosphamide doses (75, 150, 300 mg/kg) on the total white blood cell count is shown in Fig. 1. A significant leukopenia was observed in all cyclophosphamide-treated groups between 1 and 5 days after treatment. There was, in addition, a significant dose-related effect 3 days after treatment. The white cell counts began to return to normal after 5 days and approached controls (75–125%) between 7 and 13 days after treatment. The treated mice developed an eosinophilia as the white blood count returned to normal. The eosinophilia, however, was neither pathological (11) nor statistically significant. There were, in addition, no changes in the number of neutrophils, lymphocytes, or monocytes.

Cyclophosphamide (75 mg/kg) was administered to phenobarbital-pretreated, SKF 525-A-pretreated, or nonpretreated mice. Figure 2 shows the leukopenic effect of cyclophosphamide in pretreated mice relative to its leukopenic effect in nonpretreated mice. Phenobarbital pretreatment significantly increased cyclophosphamide leukopenia 2 days after treatment. SKF 525-A pretreatment, on the other hand, significantly reduced the drug-induced leukopenia at 4 and 6 days after treatment. Mice pretreated with both phenobarbital and SKF 525-A had significantly lower white blood cell counts than nonpretreated mice 16 days after treatment. This delayed effect is probably not due to a direct action of cyclophosphamide since 80 to 90% of a cyclophosphamide dose is eliminated within 24 hr of administration (15). There were no significant differences in the white blood cell counts between nontreated groups and groups treated only with phenobarbital or SKF 525-A.

Discussion. Phenobarbital-pretreated mice had a significantly greater cyclophosphamide activating ability than controls. SKF 525-A pretreatment, on the other hand, significantly reduced the ability of mice to activate the parent compound (Table I). The dose-

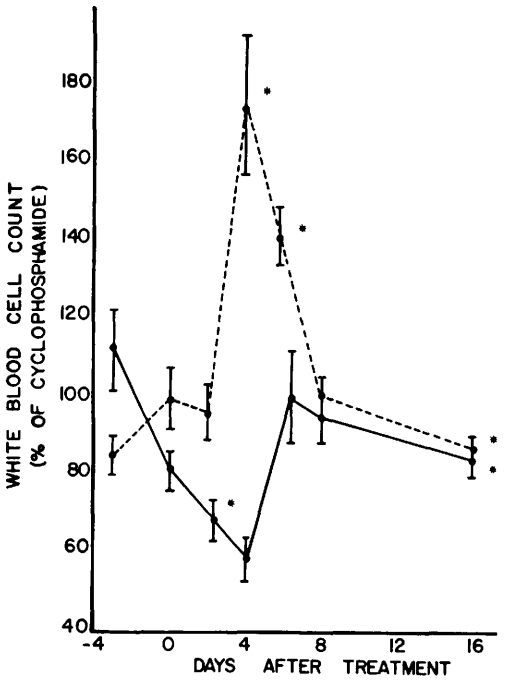

FIG. 2. Cyclophosphamide-induced leukopenia in phenobarbital (—)-; and SKF 525-A (- - -)-pretreated mice relative to nonpretreated mice as a function of days after cyclophosphamide treatment: The values plotted represent the mean percentage of control value ± SE for 5 determinations. The control value was determined at each of the indicated times after treatment. The pretreated group receiving cyclophosphamide was significantly different ($p < .05$) from a group receiving only cyclophosphamide.

related leukopenic effect of cyclophosphamide (Fig. 1) was significantly altered in pretreated mice (Fig. 2). Since phenobarbital increased, and SKF 525-A decreased, the ability of a given cyclophosphamide dose to depress the white blood cell count, it was concluded that the drug-induced leukopenia was produced by metabolites rather than the parent compound. Alkylating metabolites, therefore, have been associated with cyclophosphamide-induced leukopenia in addition to drug-induced lethality and cytotoxicity in tissue cultures (see Introduction).

Cyclophosphamide teratogenicity in mice, on the other hand, was correlated with the parent compound rather than metabolites (9, 15). Cyclophosphamide neonatal toxicity in mice, in addition, was shown to occur at a time when the animal has a poorly developed ability to convert parent compound to alkylating metabolites (10, 16, 17). These observations suggest that the parent compound has unique toxic effects in developing animals. The mouse, therefore, offers a system in which it is possible to study the toxic effects of both the parent compound and its metabolites.

Summary. Cyclophosphamide (75, 100, and 300 mg/kg, ip) produced a significant decrease in total white blood cell counts between 1 and 5 days after drug treatment. There was a significant dose-related effect 3 days after treatment. Phenobarbital pretreatment significantly increased both the *in vitro* production of alkylating metabolites and cyclophosphamide-induced leukopenia 2 days after treatment. SKF 525-A pretreatment significantly reduced both the *in vitro* formation of alkylating metabolites and the drug-induced leukopenia at 4 and 6 days after treatment. These observations indicate that alkylating metabolites of cyclophosphamide are responsible for the dose-related leukopenic effect of this antineoplastic agent.

This research was supported by Grant No. GRS 71-0955 from Michigan State University College of Human Medicine. One of us (R.D.S.) was supported by NIH Training Grant No. GM 01761).

1. Arnold, H., and Bourseaux, F., Angew, Chem. **70**, 539 (1958).

2. Brock, N., and Hohorst, H. J., Arzneim.-Forsch. **13**, 1021 (1963).

3. Foley, G. E., Friedman, O. M., and Drolet, B. P., Cancer Res. **21**, 57 (1961).

4. Rauen, H. M., and Kramer, K. P., Arzneim.-Forsch. **14**, 1066 (1964).

5. Dixon, R. L., J. Pharm. Sci. **57**, 1351 (1968).

6. Dixon, R. L., Proc. Soc. Exp. Biol. Med. **127**, 1151 (1968).

7. Cohen, J. L., and Jao, J. Y., J. Pharmacol. Exp. Ther. **174**, 206 (1970).

8. Hart, L. G., and Adamson, R. H., Arch. Int. Pharmacodyn. Ther. **180**, 391 (1969).

9. Gibson, J. E., and Becker, B. A., Teratology **1**, 393 (1968).

10. Short, R. D., and Gibson, J. E., Toxicol. Appl. Pharmacol. **19**, 103 (1971).

11. Green, E. L. (ed.), "Biology of the Laboratory Mouse." McGraw-Hill, New York (1966).

12. Friedman, O. M., and Boger, E., Anal. Chem. 33, 906 (1961).

13. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. 193, 265 (1951).

14. Steele, R. G. D., and Torrie, J. H., "Principles and Procedures of Statistics." McGraw-Hill, New York (1960).

15. Gibson, J. E., and Becker, B. A., J. Pharmacol. Exp. Ther. 177, 256 (1971).

16. Nordlinder, H., Experientia 25, 1296 (1969).

17. Short, R. D., and Gibson, J. E., Experientia 27, 805 (1971).

Received Sept. 1, 1971. P.S.E.B.M., 1972, Vol. 139.