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Effect of Chloramphenicol on the Metabolism and Lethality of Cyclophosphamide in Rats* (32895)

ROBERT L. DIXON (Introduced by Ted A. Loomis)
Department of Pharmacology, School of Medicine, University of Washington, Seattle, Washington 98105

Cyclophosphamide Cytoxan, *N,N*-bis (β-chloroethyl)-N′, *O*-propylene phosphoric acid ester diamine monohydrate, NSC 26271) is a potent antineoplastic agent of the nitrogen mustard class and has been used in the treatment of patients with many types of neoplastic diseases (1). In contrast to the nitrogen mustards ordinarily used in cancer chemotherapy, cyclophosphamide is inert when placed in direct contact with bacteria, leukocytes, and most tumor cells in culture. Its action depends on *in vivo* activation which occurs in the liver and perhaps in other sites (2,3). The subcellular site of enzymic activation in the liver is the endoplasmic reticulum. The enzyme system which activates cyclophosphamide has been demonstrated to be affected by a number of factors that alter the microsomal metabolism of various drugs. Examples are phenobarbital (4) or chlordane (5) induced stimulation of microsomal enzymes and SKF 525-A (β-diethylaminoethyldiphenylpropylacetate) inhibition of the enzyme system (6).

This paper reports the effect of chloramphenicol (Chloromycetin), an antibiotic which is also an effective inhibitor of microsomal drug metabolism in rodents (7), on the formation of alkylating substance from cyclophosphamide *in vivo* and *in vitro* and toxicity after cyclophosphamide treatment.

Methods. Cyclophosphamide (Cytoxan) and chloramphenicol sodium succinate (Chloromycetin) were obtained commercially.

The active metabolite of cyclophosphamide was estimated by the spontaneous alkylation of γ-(4-nitrobenzyl)-pyridine (NBP reagent) (8, 9). Urine was collected from rats after treatment with cyclophosphamide and chloramphenicol or saline. Plastic metabolism cages were used, the urine was obtained, the cages were rinsed with distilled water, and the urine and rinsings were brought to a final volume of 50 ml with distilled water. Triplicate 3.0-ml aliquots were taken from each total volume for assay of alkylating material. Plasma (1.0 ml) was obtained from blood taken from anesthetized rats by cardiac puncture. The plasma was acidified with 0.2 ml of 1 *N* HCl and heated in a boiling water bath for 3 min. Three ml of distilled water were added, the solutions were mixed, and then centrifuged for 15 min prior to taking a 3.0-ml aliquot for assay of active metabolite.

In the *in vitro* metabolic studies of microsomal metabolism, 5.0 ml of 6.6% trichloroacetic acid was added to incubation beaker to stop the reaction and precipitate the protein at the end of the incubation period. The solutions were mixed and poured into a heavy walled centrifuge tube and centrifuged prior to taking a 3.0-ml aliquot for assay of material which would react with the NBP reagent. Hepatic supernatant fraction (9000*g*) provided the enzymes. The conditions of *in vitro* metabolism provided a NADPH (TPNH) generating system and an atmosphere of oxygen at 37°C. These conditions of incubation, cofactors and concentrations have been previously described (10). The substrate concentration of cyclophosphamide was 10 mg/5.0-ml incubation volume (8.7×10^{-3} *M*).

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TABLE I. Effect of Chloramphenicol on the *in Vivo* Activation of Cyclophosphamide.

	Chloramphenicol treated	Saline treated
Alkylating metabolite in urine[a]	4.584 (4.200, 4.967)	11.575 (12.400, 10.750)
Alkylating material in plasma[b]	0.484 ± 0.080	0.623 ± 0.105

[a] Urine was collected from four groups of three animals each for 90 min. Two groups received chloramphenicol 100 mg/kg 30 min prior to cyclophosphamide. Two groups received a pretreatment of saline. The values presented are means of the total amount of alkylating material in a 3.0-ml aliquot of urine with the two individual values presented in parentheses.

[b] Values presented are mean ± SD OD readings of the alkylating material recovered from 1.0 ml of plasma 90 min after cyclophosphamide treatment. These values are significantly different at the $p = 0.05$ level.

In the *in vivo* studies, adult male rats were treated subcutaneously with chloramphenicol or saline and then received cyclophosphamide orally. All drugs were administered as an aqueous solution such that animals received 0.001 ml for each gram of body weight for subcutaneous treatment and 0.01 ml of solution for each gram of body weight for oral administration. In all lethality studies, the animals were observed daily for 21 days.

Statistical methods used are described by Snedecor (11) and LD_{50} estimates and potency ratios were determined by the method of Litchfield and Wilcoxon (12).

Results. Adult male rats treated with chloramphenicol (100 mg/kg) subcutaneously prior to treatment intraperitoneally with cyclophosphamide (500 mg/kg) excreted 60% less of a metabolite capable of alkylation in 90 min than did animals pretreated with saline subcutaneously. These data are presented in Table I. Urine was collected from four groups of three animals (2 control, 2 treated) for the first 90 min following cyclophosphamide treatment using individual plastic metabolism cages. The collecting funnel was rinsed with distilled water. Urine and cage rinsings were brought to a total volume of 50 ml. A 3.0-ml aliquot was taken for assay of material capable of alkylating the NBP reagent. In the final step of this assay the reacted metabolite from the chloramphenicol treated group was extracted into 10 ml of nonpolar solvent and read on a colorimeter. The reacted metabolite in the urine of saline pretreated animals after cyclophosphamide was extracted into 15 ml of nonpolar solvent prior to reading. Table I presents the total OD readings (reading × dilution) obtained after analysis of the 3.0-ml urine sample.

At the end of the 90-min collection period, a blood sample was taken from each animal (6 control, 6 treated) after ether anesthesia. One ml of plasma was prepared as described in *"Methods"* and an aliquot was analyzed for alkylating material. In this case the reacted metabolite in each aliquot was extracted into 5.0 ml of nonpolar solvent before colorimetric determination. These data are presented in Table I and reveal a significant difference in the amount of circulating metabolite capable of alkylation when the two groups are compared. The chloramphenicol treated animals had less alkylating material in their plasma than saline treated controls.

Chloramphenicol was capable of inhibiting the *in vitro* metabolism of cyclophosphamide 50% at an extrapolated value of 1.12×10^{-3} *M*. The actual experimental values are presented in Table II. Thus, chloramphenicol appears to affect the metabolism of cyclophosphamide at about the same concentration range that it inhibits the metabolism of such drugs as hexobarbital, acetanilid, codeine, and aminopyrine (7).

Chloramphenicol increased both the day of mean death and the mean lethality of cyclophosphamide. Table III demonstrates this effect. It can be seen that animals pretreated with chloramphenicol survived longer and in greater number than animals pretreated with saline. These data indicate that chloramphenicol is capable of partially protecting

TABLE II. Effect of Chloramphenicol on the *in Vitro* Metabolism of Cyclophosphamide.

Concentration of chloramphenicol ($\times 10^{-6} M$)	Metabolism[a] (%)
None	100
7.8	93
15.6	77
39.0	72
78.0	60

[a] The 5.0-ml incubation mixture (see "*Methods*") was mixed with 5.0 ml of 6.6% trichloroacetic acid and centrifuged. A 3.0-ml aliquot of supernatant was assayed for alkylating material as described and the color extracted into 4.0 ml of nonpolar solvent prior to colorimetric reading. The mean control reading was 0.460.

TABLE III. Effect of Chloramphenicol on Percentage Dead and Day of Death after Cyclophosphamide.[a]

Treatment:	Accumulative percentage dead			
	Cyclophosphamide (300 mg/kg)		Cyclophosphamide (200 mg/kg)	
Pretreatment:	Saline	Chloramphenicol	Saline	Chloramphenicol
Days after treatment				
4	0	0	0	0
5	20	0	0	0
6	80	20	10	0
7	100	40	40	0
8	—	50	60	0
9	—	50	70	0
10	—	50	80	20
11	—	50	80	30
12	—	60	80	30
13	—	60	80	30
14	—	60	90	30
15–19	—	60	90	30
20	—	70	90	30
21	—	70	90	30

[a] Each group of 10 animals was treated with either chloramphenicol (100 mg/kg) or saline 30 min prior to receiving the dose of cyclophosphamide indicated. The pretreatment was by the subcutaneous and the treatment via the oral route.

against the toxic and lethal effects of cyclophosphamide.

Chloramphenicol pretreatment significantly reduced the relative potency of cyclophosphamide. Dose response curves for each treatment are presented in Fig. 1. These dose response curves were drawn with data that was not significantly heterogeneous and, thus, fit the line well. The slopes were also demonstrated to be parallel and the relative potency estimated to be 1.52 (1.13–2.05). The individual LD_{50} values after oral administration of cyclophosphamide were 165 (145–188) mg/kg for the saline preatreated group and 250 (192–325) mg/kg for the chloramphenicol pretreated group. These values are significantly different.

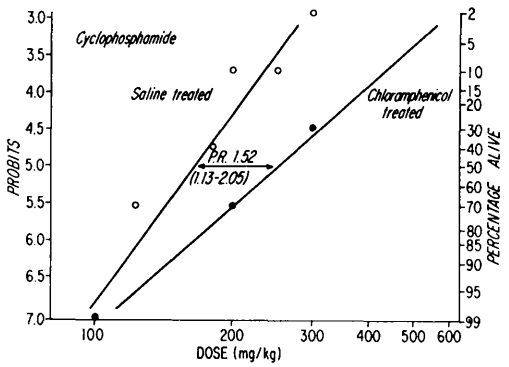

FIG. 1. Lethality of cyclophosphamide to rats pretreated with either saline or chloramphenicol (100 mg/kg sc). Each point represents at least 10 animals. The potency ratio of 1.52 indicates a significant difference between the two groups.

Discussion. Chloramphenicol pretreatment has been demonstrated to alter the lethality of cyclophosphamide *in vivo* resulting in a longer time for mean death (LT_{50}) and a higher dose calculated to be lethal to half of the animals treated (LD_{50}). This clinically useful antibiotic was also capable of decreasing the production of a metabolite capable of alkylation (active metabolite) after cyclophosphamide treatment *in vivo*. Chloramphenicol treatment resulted in a decrease in the amount of activated material excreted in 90 min, and a lower level of activated product in the circulation. Chloramphenicol also inhibited the *in vitro* production of metabolites capable of alkylation after 30-min incubation at 37°C of hepatic supernatant fraction with a NADPH (TPNH) generating system. Cyclophosphamide was the substrate and the in-

cubation was carried out under oxygen at 37°C. Chloramphenicol at 1.12×10^{-3} *M* was able to inhibit this system 50%.

Chloramphenicol is a broad spectrum antibiotic which appears to act by interfering with the synthesis of complete proteins but does not appear to affect intermediate steps in amino acid and peptide metabolism or nucleic acid synthesis. The bacteriostatic effect is thought to involve the attachment of mRNA to ribosome. The clinical usefulness of chloramphenicol is somewhat limited by a poorly understood toxic effect of bone marrow depression. Rarely, a true agranulocytosis and aplastic anemia develop which are not reversible and generally are fatal. This reaction is estimated to occur once in every 50,000–100,000 cases (13).

Cyclophosphamide is widely used in the treatment of disseminated or nonresectable neoplastic diseases such as leukemias, lymphomas, multiple myeloma, and some carcinomas. The drug is administered in an inactive form which depends on *in vivo* biotransformation to produce the active alkylating agent. Thus, drugs which inhibit microsomal drug metabolism could decrease the therapeutic effectiveness of cyclophosphamide and other agents which stimulate these microsomal enzyme systems, e.g., phenobarbital (5) and chlordane (6) could enhance the activity of the drug and perhaps result in greater toxicity.

The mechanism by which chloramphenicol decreases the lethality of cyclophosphamide is most likely due to inhibition of microsomal drug metabolizing enzymes necessary for the conversion of cyclophosphamide to its active form. The dose of chloramphenicol used was relatively high (100 mg/kg) but obviously did not have a lethal effect as the chloramphenicol group of animals were protected from the lethal effect of cyclophosphamide. The possibility that chloramphenicol provides protection as a result of its antibacterial action seems remote. The chloramphenicol was administered only once, and no animals in either group died for a period of 4 days after treatment. Thus, during the toxic phase of the drug response no chloramphenicol was present to provide protection.

These results further indicate the clinical importance of a recognition of drug interactions. It appears possible that treatment with chloramphenicol during a concomitant cyclophosphamide regimen could alter drug effectiveness and perhaps lessen the therapeutic efficacy of the drug. This would be in contrast to what might be observed after enzyme stimulation due, for example, to chlordan. In this case, more of the active metabolite would be formed and the treatment would result in greater therapeutic and perhaps toxic effect. This demonstrated decreased effectiveness of cyclophosphamide after inhibition of microsomal drug metabolizing enzymes might also provide protection in the case of accidental drug overdosage in the clinic.

Summary. Chloramphenicol pretreatment reduced the lethality of cyclophosphamide *in vivo* and resulted in an increased time of mean death and a higher mean lethal dose. Chloramphenicol pretreatment decreased the *in vivo* conversion of cyclophosphamide to a metabolite capable of alkylation. After pretreatment with chloramphenicol, a decreased amount of activated product appeared in the urine during 90 min and blood levels of active metabolite were less when measured after 90 min. *In vitro* chloramphenicol decreased the metabolism of cyclophospamide 50% at approximately 10^{-3} *M*. It is concluded that the protection afforded animals by chloramphenicol after treatment with lethal levels of cyclophosphamide is due to an inhibition of microsomal drug-metabolizing enzymes which are responsible for the *in vivo* activation of cyclophosphamide.

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Prolonged Catecholamine-Dependent Cardioaccelerator Action of Bretylium (32896)

S. S. CHEN[1] (Introduced by W. B. Youmans)

Department of Physiology, University of Wisconsin, Madison, Wisconsin 53706

Adrenomimetic manifestations following the administration of bretylium have been demonstrated in several species of animals and in different types of experimental conditions. These studies have been reviewed by Boura and Green (1), and by Holtz and Palm (2). It is generally agreed that the adrenomimetic effect of bretylium is due to the release of norepinephrine from adrenergic nerve endings because it occurs in isolated tissues (3) and adrenalectomized animals (4); it does not occur in tissues depleted of catecholamines by reserpine, and it may reappear if the reserpinized preparation is treated with norepinephrine (3,5–7). Also the adrenomimetic responses following bretylium can be blocked by appropriate adrenergic blocking agents (5,7). The duration of adrenomimetic effect following injection of bretylium is said to be 20–30 min, as judged from the change in blood pressure, heart rate, and the contractility of the heart (5,8). The results obtained in these studies of effects on heart rate in dogs with baroceptor reflexes inactivated indicate that adrenomimetic effects continue considerably longer than has been reported previously.

Method. Dogs weighing 9–15 kg were premedicated with morphine (3 mg/kg) and anesthetized by intravenous injection of α-chloralose (100 mg/kg) or pentobarbital (20 mg/kg). In order to suppress the influences of buffering reflexes, tetraethylammonium chloride (TEAC) was administered in doses of 20–25 mg/kg intravenously and, at the same time, an additional 40–50 mg/kg intramuscularly. The TEAC produced an initial rise in the heart rate, and 40–50 min later the heart rate became stable. Then bretylium was injected. The heart rate was determined from lead II of the electrocardiogram. The number of cardiac cycles/10-sec period, including fractions, was counted and the rate expressed as beats/min. Blood pressure was measured by a Statham pressure transducer connected by polyethylene tubing to a needle in the femoral artery. All recordings were made on a Gilson polygraph.

Vagotomized spinal dogs were prepared by transecting the spinal cord at the level of C_2 and cutting vagi at the middle cervical level. Respiration was maintained by a Harvard respirator. After the heart rate and blood pressure became stable bretylium was injected.

For the studies of urinary catecholamine output urine was collected from female dogs by catheterization. The experiment was divided into four periods. The first period was the 45-min period after TEAC administration and before bretylium injection. The second period was the first 20 min after bretylium injection and the third and fourth periods were from 20–80 and from 80–180 min, respectively, after bretylium injection. Epinephrine and norepinephrine in urine were determined by the method of Anton and Sayre (9) using Aminco spectrophosphorimeter–fluorometer.

Results. Bretylium tosylate, in doses of 10

[1] Supported by Wisconsin Heart Association.