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EFFECTS *IN VIVO* OF LYMPHOMA ASCITES TUMORS AND PROCARBAZINE, ALONE AND IN COMBINATION, UPON HEPATIC DRUG-METABOLIZING ENZYMES OF MICE

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Abstract—Studies have been conducted on hepatic microsomal enzymes after treatment of two strains of male mice, BDF_1 and DBA/2J, each with a single dose (300 mg/kg body weight) of procarbazine (PCZ) hydrochloride alone or in combination with a 6- to 7-day prior implantation of murine leukemic lymphoma L5178Y ascites cells. Mice were sacrificed at 4, 8 or 16 hr after i.p. injection of PCZ and found to possess levels of microsomal aniline hydroxylase, ethylmorphine *N*-demethylase, nitrobenzoate reductase activities and cytochrome P-450 content which were depressed to 78, 76, 54 and 61 per cent of untreated controls, respectively, for BDF_1 mice and 61, 51, 39 and 51 per cent of untreated controls, respectively, for DBA/2J mice. Mice implanted with lymphoma cells and sacrificed 6–7 days later without PCZ treatment had hepatic microsomal enzyme activity levels which were depressed to about the same extent as those receiving PCZ treatment only. PCZ treatment 6–7 days after lymphoma implantation caused severe depression of microsomal enzyme activities in both mouse strains. The maximum depressions expressed as per cent of untreated controls were: cytochrome P-450, 33 per cent; aniline hydroxylase, 32 per cent; ethylmorphine *N*-demethylase, 33 per cent; and nitrobenzoate reductase, 33 per cent. As a possible explanation for the PCZ effects, it is proposed that PCZ is serving as a tightly bound competitive substrate to cytochrome P-450-related enzyme systems.

Neubert and Hoffmeister [1] compared the ability of rat liver microsomes and those from rat hepatomas to oxidize drugs. Oxidation of side chains, hydroxylation and various *O*- and *N*-demethylations were mostly abolished in the hepatoma microsomes. Impairment of drug metabolism in the liver of non hepatoma tumor-bearing rats was described by Kato *et al.* [2]. Subsequent observations by Kato *et al.* [3, 4] and other workers [5–11] on drug and steroid metabolism of tumor-bearing animals suggested more than one mechanism for tumor-related impairment of drug metabolism. Workers including Tardiff and Dubois [12], Donelli *et al.* [13], Donelli and Garattini [14], Eade *et al.* [15], Thompson and Larson [16], Lu [17] and Klubes and Cerna [18] have reported the alteration of drug metabolism by certain antitumor drugs in normal animals. Little is known about possible alterations of liver function during cancer chemotherapy: specifically, the combined effects of antineoplastic drugs and tumors upon drug metabolism by the liver.

In this paper, we wish to report the combined effects *in vivo* of treatment with procarbazine (PCZ) hydrochloride (Matulane) and i.p. implantation of lymphoma ascites cells, L5178Y, upon drug metabolism by liver microsomes prepared from mice. Nearly a 3-fold decrease in certain microsomal drug-metabolizing enzymic activities and cytochrome P-450 content has been observed by assays *in vitro* of livers of treated mice in comparison with livers of untreated control mice.

EXPERIMENTAL

The L5178Y lymphoma cell line was kindly furnished by Dr. Alan Sartorelli, Yale University. The cell line was maintained by transplantation weekly [19] into 20-g male BDF_1 mice (Texas Inbred Mice Co., Houston, Tex.) or DBA/2J male mice (Jackson Laboratories, Bar Harbor, Me.). Procarbazine hydrochloride, a gift of Hoffmann-LaRoche, Nutley, N.J., was administered intraperitoneally at a dose of 300 mg/kg body weight in glass-distilled water. Control animals were injected with saline. Lymphoma ascites tumors were measured as total packed cell volume [20]. Lymphoma-bearing mice at 6–7 days after i.p. implantation of lymphoma ascites cells had a cell titer of $2{\cdot}0 \pm 0{\cdot}4 \times 10^8$/ml of i.p. fluid. PCZ was administered at that time, and changes in liver microsomes were observed at 4, 8 and 16 hr. Lymphoma controls (0 time) were injected with saline. At 16 hr after PCZ treatment, a 10–20 per cent decrease in ascites cell titer was noted when compared to lymphoma-bearing control mice.

Liver microsomes were prepared according to the procedure of Borton *et al.* [21]. Protein concentration of the microsomes was determined by the method of Lowry *et al.* [22] and the cytochrome b_5 and P-450 contents were determined spectrophotometrically at room temperature by the $\Delta E_{427} - E_{410}$ and the $\Delta E_{450} - E_{410}$ according to the methods of Smuckler *et al.* [23].

The reduction of *p*-nitrobenzoic acid to *p*-amino-

benzoic acid was based on a modified method of Smith and Van Loon [24]. Incubation mixtures in 10-ml flasks consisted of 8 mg protein, 1 μmole NADP, 25 μmoles glucose 6-phosphate, 1·2 units glucose-6-phosphate dehydrogenase, 25 μmoles $MgCl_2$, 28 μmoles nicotinamide, 12 μmoles *p*-nitrobenzoic acid and adjusted to a final volume of 2·85 ml with 0·1 M, pH 7·4, phosphate buffer. The samples were capped with serum vial stoppers, flushed for 2 min with nitrogen gas and then incubated for 20 min at 37° in a shaking waterbath. The reaction was stopped with 2·5 ml 15% trichloroacetic acid and the mixture was centrifuged at room temperature. Three ml supernatant was removed and treated as follows: 0·5 ml of 0·2% sodium nitrite was added and after 3 min, 0·5 ml of 1% ammonium sulfamate was added. Three min later, 0·5 ml of 0·2% *N*-(1-naphthyl) ethylenediamine dihydrochloride was added and after 15 min, 2 ml of 95% ethanol was added to stabilize the color, which was measured at 540 nm.

The hydroxylation of aniline was determined by measuring the formation of *p*-aminophenol according to the method of Kato and Gillette [25]. In our assay, 20 μmoles redistilled aniline was incubated with the reaction mixture for 20 min at 37 in a shaking waterbath.

The *N*-demethylation of ethylmorphine was determined by measuring formaldehyde formation. The incubation mixture consisted of 2 mg protein, 2 μmoles NADP, 50 μmoles glucose 6-phosphate, 2 units glucose-6-phosphate dehydrogenase, 25 μmoles $MgCl_2$, 3 μmoles ethylmorphine and adjusted to a final volume of 3 ml with 0·1 M, pH 7·4, phosphate buffer. The mixtures were incubated and analyzed for formaldehyde with a modified Nash reagent according to the method of Davies *et al.* [26].

All data presented represent mean values ± standard deviations of the sample population from at least four experiments in which the livers of two mice were pooled prior to microsome preparation. Calculations were done with an Olivetti Programma 101 and a statistical program by Williams [27].

RESULTS

Liver weights of control, PCZ-treated or PCZ plus lymphoma-treated mice did not change significantly during the 4-, 8-, or 16-hr intervals after PCZ treatment. Therefore, any changes in cytochrome P-450 content or enzyme activites were the same percentage changes when expressed as either per mg of microsomal protein or per total liver weight.

Table 1. Activity of enzymes and cytochrome P-450 content of liver microsomes from normal BDF_1 and DBA/2J mice

	BDF_1	DBA/2J
Cytochrome P-450 (nmoles/mg protein)	0·66 ± 0·12	0·72 ± 0·13
Ethylmorphine demethylase (nmoles/min/mg protein)	11·7 ± 2·7	13·6 ± 2·4
Aniline hydroxylase (nmoles/min/mg protein)	0·74 ± 0·16	0·99 ± 0·18
Nitroreductase (nmoles/min/mg protein)	0·31 ± 0·05	0·48 ± 0·01

Effect of prior treatment with PCZ on liver microsomes. Liver microsomes prepared from two strains of male mice, BDF_1 and DBA/2J, were assayed before (Table 1) and after i.p. treatment with PCZ (300 mg/kg body weight). Comparison was made with microsomes prepared from control mice injected with saline. PCZ treatment alone did not cause a significant decrease in hepatic microsomal protein during 4-, 8- and 16-hr intervals after treatment (Table 2).

The cytochrome P-450 content of the liver microsomes was decreased in both mouse strains by PCZ treatment (Fig. 1). DBA/2J mice treated with PCZ had a liver cytochrome P-450 content which was decreased 50 per cent after 16 hr when compared to that of untreated control mice (zero time, Fig. 1). Similarly, PCZ treatment of BDF_1 mice resulted in a decrease to 61 per cent of the microsomal cytochrome P-450 content of untreated control mice (zero time) 8 hr after treatment (Fig. 1). Ethylmorphine *N*-demethylase activity showed a similar pattern with PCZ treatment causing decreases of 55–62 per cent of untreated controls (zero time) with DBA/2J mice. Again BDF_1 mice showed a lesser effect of PCZ treatment on this enzyme activity. Maximum effect was at 4 and 8 hr (76 per cent of untreated controls) and then activity returned to 106 per cent of untreated controls at 16 hr. BDF_1 mouse microsomes showed a maximum decrease in nitroreductase activity at 4 hr (54 per cent of untreated controls) with a gradual recovery.

Table 2. Protein content of liver microsomes after procarbazine treatment *in vivo* of normal and tumor-bearing BDF_1 and DBA/2J mice

Mouse strain	Controls	Procarbazine only Time after procarbazine treatment 4 hr	8 hr	16 hr
			(mg/g liver wt)	
BDF_1	9·7 ± 1·4	7·9 ± 0·36	8·5 ± 1·1	8·8 ± 1·3
DBA/2J	10·4 ± 1·1	10·7 ± 0·05	10·4 ± 0·30	8·80 ± 2·6
	Lymphoma controls		Lymphoma + procarbazine (mg/g liver wt)	
BDF_1	7·30 ± 1·2	7·80 ± 1·0	6·90 ± 0·41	7·50 ± 1·2
DBA/2J	9·30 ± 1·3	8·30 ± 1·4	8·60 ± 1·3	8·40 ± 1·0

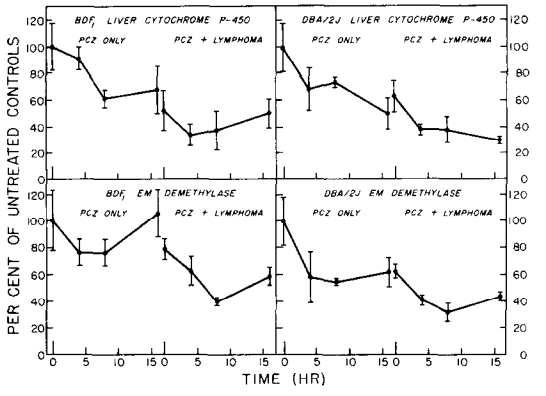

Fig. 1. Effects of prior treatment of normal or tumor-bearing mice, BDF_1 and DBA/2J, with procarbazine (PCZ) on liver microsomal cytochrome P-450 content and ethylmorphine (EM) demethylase enzyme activity. Zero time for mice treated with PCZ only are saline-injected controls. Zero time for mice treated with PCZ plus lymphoma cells are control mice implanted 6–7 days prior to sacrifice but not given PCZ. All mice sacrificed at 4, 8 and 16 hr were given a single dose of PCZ. Other experimental details are given in the text.

Aniline hydroxylase activity was decreased to 69, 74 and 51 per cent of untreated controls at 4-, 8- and 16-hr, respectively, after PCZ treatment of DBA/2J mice. Treatment of BDF_1 mice caused this enzyme activity to be decreased to 78 and 76 per cent of untreated controls at 8 and 16 hr respectively.

Effects of lymphoma ascites tumors on liver microsomes. The presence of 6- to 7-day-old ascites tumors resulted in mouse liver cytochrome P-450 content and enzyme activities being reduced in nearly every instance to an amount equal to the maximum extent caused by PCZ (Figs. 1 and 2). Cytochrome P-450 was reduced to 53 and 64 per cent of untreated controls for BDF_1 and DBA/2J mice respectively (Fig. 1). Similarly, microsomal enzyme activities measured were depressed to levels which ranged from 48 and 50 per cent of untreated controls for nitroreductase, to 55 and 62 per cent of untreated controls for aniline hydroxylase and to 79 and 63 per cent of untreated controls for EM demethylase in BDF_1 and DBA/2J strains respectively (Figs. 1 and 2).

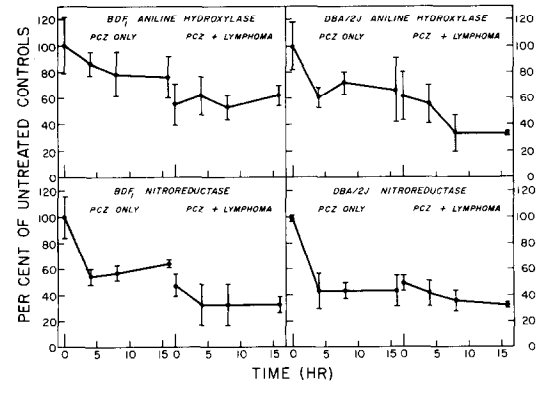

Fig. 2. Effects of prior treatment of normal or tumor-bearing mice, BDF_1 and DBA/2J, with PCZ on liver microsomal aniline hydroxylase and nitroreductase enzyme activities. Zero time controls are identical to those described in Fig. 1. Other experimental details are given in the text.

Combined effects of PCZ and lymphoma ascites tumors on liver microsomes. The combined effects of lymphoma ascites and PCZ significantly decreased the microsomal protein content of the livers of both mouse strains when compared to that of untreated mice. The maximum decrease was to 80 and 71 per cent of untreated controls for DBA/2J and BDF_1 mice respectively (Table 2). Liver cytochrome P-450 content and all measured enzyme activities were decreased further by the combined effects of PCZ treatment and ascites tumors than by either single effect (Figs. 1 and 2). While the effects were not additive, the combination of PCZ and ascites tumors caused decreases up to 3-fold. Extreme decreases observed were: DBA/2J cytochrome P-450 content 31 per cent of untreated controls 16 hr after PCZ; nitroreductase 33 per cent of controls in both mouse strains; and DBA/2J EM demethylase and aniline hydroxylase activites at 32 and 34 per cent of controls at maximum decreases. Tumor-bearing BDF_1 mice demonstrated a slightly greater trend than tumor-bearing DBA/2J mice to recover both cytochrome P-450 content and enzymic activity by the end of 16 hr after PCZ. DBA/2J mice showed a slight recovery of only EM demethylase activity, while BDF_1 mice regained some activity with aniline hydroxylase and EM demethylase but not nitroreductase.

DISCUSSION

PCZ treatment alone at a dose level of 300 mg/kg body weight caused a significant decrease in not only the content of cytochrome P-450 of liver in both mouse strains but also in the activity of the three microsomal enzymes assayed when compared to microsomes prepared from untreated mice. Eade *et al.* [15] reported that PCZ significantly prolonged pentobarbital sleeping time of rats. Various other hydrazines have been reported to inhibit drug metabolism *in vivo* in rats (Kato *et al.* [28]). Comparing effects *in vivo* with microsome inhibitions *in vitro*, these workers concluded that the lipid solubility of hydrazines and hydrazides was more important than their chemical configuration in causing inhibition of drug-metabolizing enzymes of liver.

Hydrazines could be inhibitors by serving as substrates for certain microsomal enzyme systems. Prough *et al.* [29, 30] and Wittkop *et al.* [31] have demonstrated the microsomal conversion of the *N*-methyl group of PCZ to both formaldehyde and methane by rat liver microsomes. It is possible that a chemical reactive intermediate is formed from the remainder of the PCZ molecule after *N*-demethylation. A possible product is either a benzylhydrazine or benzyldiazene derivative (Prough *et al.* [30]). Further metabolism of such intermediates would likely proceed via a free radical intermediate which could cause alteration of the site of metabolism. The decrease in microsomal enzyme activity is in agreement with the observation that PCZ undergoes a type II binding to cytochrome P-450 [31]. Prough [32] has reported that the *N*-oxidation of methylhydrazine, 1,1-dimethylhydrazine, 1,2-dimethylhydrazine

and PCZ is catalyzed by purified microsomal mixed-function amine oxidase.

Implantation of mice with lymphoma L5178Y ascites cells followed by 6–7 days tumor growth resulted in decreased levels of cytochrome P-450 and microsomal enzyme activity. These results are in agreement with those of workers including Kato *et al.* [2], Kato and Takahashi [4] and Rosso *et al.* [33]. These workers have proposed that serum constituents, such as toxohormone [34], may play a role in such impairment and that surgical removal of the tumor abolishes the impairment of drug metabolism [33]. Further work is necessary to determine if such a mechanism is important in the results reported here. In this report we have attempted to determine to what degree the combined effects of PCZ treatment and implantation of mice with tumors would alter the impairment of hepatic drug metabolism. The evidence presented here suggests that the impairment caused by the tumor is neither abolished nor reduced upon PCZ treatment but in some instances actually enhanced by almost an additive amount depending upon the mouse species. The DBA/2J strain has fewer (generally 2–3 days) days of life expectancy after lymphoma implantation than the BDF_1 strain. Data presented here indicate a greater impairment of microsomal enzyme activity and a greater decrease in cytochrome P-450 content is observed in the DBA/2J strain of mice than in the BDF_1 mice.

Recently Klubes and Cerna [18] observed that pretreatment of rats with a single dose of 5-fluorouracil (5-FU) decreased hepatic drug-metabolizing enzyme activities 7 days later. Using the 10,000 *g* supernatant fraction from liver homogenates, aniline hydroxylase, ethylmorphine *N*-demethylase and 2-(methylthio)-benzothiazole *S*-demethylase activities were depressed 41, 40 and 25 per cent, respectively, while the protein content was unchanged. On day 14 after 5-FU treatment, enzyme activities were unchanged. The authors concluded that the depression of enzyme activities on day 7 may be due to the direct action of the drug on the synthesis and/or turnover of these enzymes, delayed growth, or a combination of these effects.

Thus, much is yet to be understood about the effects of tumors and antitumor drugs upon hepatic drug metabolism. Since procarbazine is administered daily to human patients during its clinical use in cancer chemotherapy for periods generally ranging from 14 to 35 days [35], this study may be relevant to the clinical situation.

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