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INCREASED ANTITUMOUR ACTIVITY OF CHLORAMBUCIL FOLLOWING PRETREATMENT WITH INDUCERS OF DRUG-METABOLIZING ENZYMES

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Abstract—Alkylating agent-sensitive and resistant lines of tumour cells growing either in the rat or in suspension culture have been pretreated with inducers of microsomal drug metabolizing enzymes prior to chlorambucil. This enhanced the cytotoxicity of chlorambucil in the sensitive tumour cells but was without effect on the resistant cells. In the intact animal the drug combination of phenobarbitone and chlorambucil did not appear to be more toxic to normal bone marrow cells than chlorambucil alone.

IT HAS been demonstrated that chlorambucil, $(ClCH_2CH_2)_2NC_6H_4(CH_2)_3COOH$, is metabolized differently by two strains of tumour cells *in vitro*.[1] A strain of drug-resistant Yoshida ascites cells can hydrolyse the chloroethyl groups and modify the aromatic ring of the drug more extensively than the drug-sensitive strain from which it was derived. This finding has been confirmed in lymphocytes isolated from the peripheral blood of patients with chronic lymphocytic leukaemia. These patients may be classified in terms of this varied drug metabolism into two groups, only one of which responds well to treatment.[2]

The possible involvement of metabolism in modifying the mode of action of chlorambucil led us to investigate whether substances known to alter the levels of drug-metabolizing enzymes could influence the cytotoxic effects of this drug.[3] In this communication, we are reporting the results of experiments where pretreatment with known inducers of drug-metabolizing enzymes (phenobarbitone, 3-methylcholanthrene, Sudan III and benz[a]anthracene) has altered the subsequent response of three strains of tumour cells to treatment with chlorambucil.

MATERIALS AND METHODS

Chlorambucil (Leukeran) $(ClCH_2CH_2)_2NC_6H_4(CH_2)_3COOH$ was synthesized in the Chester Beatty Research Institute. Sodium phenobarbitone was purchased from Boots Pure Drug Co., Ltd., Nottingham, 3-methylcholanthrene from Eastman–Kodak Ltd., Sudan III from British Drug Houses Ltd., and benz[a]anthracene was obtained from Hopkin and Williams Ltd., or B.D.H. Ltd., AnalaR grades of other chemicals were used where available. Foetal bovine serum and Fisher's medium for leukaemic cells of mice were supplied by Flow Laboratories Ltd., and Grand Island Biological Co., California, respectively.

Animals. Drug-sensitive and drug-resistant strains of the Yoshida ascites sarcoma were utilized for *in vivo* experiments. For full details of animal experimentation and

tumour transplantation, see techniques previously used.[4,5] The enzyme inducers were given intraperitoneally (at doses shown in Table 1) 12 hr prior to the subcutaneous injection of the tumour-bearing animals with chlorambucil (8 mg/kg) on the fourth day following tumour transplantation. This dose of chlorambucil was known to induce regression of the sensitive tumour but was without effect on the growth-rate of the resistant tumour.[5] Each experimental point represents the mean value from four animals and each experiment was performed in duplicate.

TABLE 1. DRUG CONCENTRATIONS AND SOLVENTS USED

Drug	Solvent	Drug concn	
		In vivo (mg/kg)	*In vitro* (M)
Chlorambucil	Ethanolic–HCl–propylene glycol*	8	Dependent on cell line studied (see text)
Phenobarbitone	Isotonic saline or Fisher's medium	100	2×10^{-3}
Methylcholanthrene	Dimethylsulphoxide	20	10^{-4}
Sudan III	Arachis oil	40	
Benz[a]anthracene	Ethanol		10^{-4}

* The drug was dissolved in 1 vol. of 2% w/v HCl in ethanol and diluted with 9 vol. of phosphate–propylene glycol solution (prepared by dissolving 20 g dipotassium hydrogen phosphate and 450 ml propylene glycol in water and diluting to 1 litre).

Metabolism of chlorambucil. Tumour-bearing animals were treated with phenobarbitone (100 mg/kg, i.p.) 12 hr prior to chlorambucil (8 mg/kg, s.c.). Control groups received phenobarbitone alone, chlorambucil alone or remained without treatment. Animals were killed at the times specified in the Results section, the tumour cells harvested and *in vitro* incubations prepared. Chlorambucil was added to a concentration of 450 μg/ml to these incubates. The levels of "total" intracellular drug were then measured during a 100-min incubation at 37° by the method previously described,[1] utilizing the characteristic benzenoid absorption of the drug.

Studies in vitro. The alkylating agent-sensitive and -resistant lines of Yoshida cells and L5178Y cells were grown in suspension culture. The Yoshida cells were grown as short-term primary cell cultures: following aspiration from tumour-bearing animals, the cells were washed and resuspended in Fisher's medium containing 20% foetal bovine serum. The growth-rates of the two cell lines which were in logarithmic growth during the period of study were measured by daily haemocytometer counts.

The difference in sensitivity to chlorambucil between the two lines has been demonstrated in terms of cell viability to be approx. 30- to 40-fold.[6] This figure was established by the use of the bioassay technique developed by Connors *et al.*[7] involving exposure of ascites tumour cells *in vitro* for 1 hr at 37° to a range of chlorambucil concentrations. The viability of the ascites cells was tested by injecting a known aliquot of tumour cells intraperitoneally into recipient rats and recording the subsequent weight of the tumour. A linear relationship has been shown to exist between the number of viable cells inoculated and the subsequent weight of the tumour.[6]

The L5178Y cells were grown continuously in suspension culture in Fisher's

medium containing 10% foetal bovine serum. The sensitivity of the cells to chlorambucil was determined from dose–response curves and by colony-forming assay, according to the method of Clin and Fisher.[8]

Protein synthesis was measured by following the incorporation of [^{3}H]-L-lysine monohydrochloride (sp.act. 244 mCi/mmole at a concentration of 1 μCi/ml) into the protein precipitated by perchloric acid using a modified Schmidt–Tannhäuser procedure, after the removal of DNA and RNA.[9] Radioactivity was assayed using a toluene–phosphor scintillant in a Packard Tri Carb liquid scintillation counter, Model 3375.

DNA was estimated according to Burton,[10] RNA by the orcinol procedure[11] and protein by the method of Lowry *et al.*[12]

RESULTS

Drug metabolism studies. The uptake of "total" chlorambucil by the Yoshida tumour cells is shown in Table 2. Pretreatment with phenobarbitone did not affect the

TABLE 2. UPTAKE AND SUBSEQUENT METABOLISM OF CHLORAMBUCIL BY TUMOUR CELLS *in vitro*

Yoshida cell line	Drug treatment *in vivo*	Time after drug treatment (days)	μg Chlorambucil per 10^7 cells after incubation at 37° for 5 min and 100 min		% Loss of benzenoid absorption in 100 min
Resistant	None	1	279	110	61
		2	288	104	63
Resistant	Phenobarbitone only	1	281	110	61
		2	290	113	60
Resistant	Chlorambucil only	1	308	106	64
		2	329	140	58
Resistant	Phenobarbitone chlorambucil +	1	264	120	56
Sensitive	None	1	297	198	33
		2	290	192	34
Sensitive	Phenobarbitone only	1	288	208	28
		2	324	218	33
Sensitive	Chlorambucil only	1	452	352	22
		2	515	475	8
Sensitive	Phenobarbitone chlorambucil +	1	445	440	2

The ascites tumour cells were aspirated from tumour-bearing animals which had been variously pretreated with drug combinations (see text). The growth curves of ascites cells in tumour-bearing animals following drug treatment are shown in Fig. 1. Chlorambucil was measured by its characteristic benzenoid adsorption (see Methods). Each value represents the mean of eight determinations. The overall scatter about any point ± 10 per cent.

ability of either drug-resistant or drug-sensitive cells to modify the benzenoid absorption of chlorambucil. Treatment of tumour-bearing animals with chlorambucil alone or with combinations of chlorambucil and phenobarbitone enhanced the subsequent uptake of the mustard by the drug-sensitive cells when incubated *in vitro*. This may be attributable to the unbalanced growth and increase in cell volume which occurs

following exposure to alkylating agents.[4,5] However, after treatment with chlorambucil, or with phenobarbitone and chlorambucil, the ability of sensitive cells to modify the u.v. absorption of chlorambucil was decreased.

Studies in vivo. The growth curves of sensitive and resistant cells in tumour-bearing animals following drug treatment are shown in Fig. 1. The growth rate of both strains

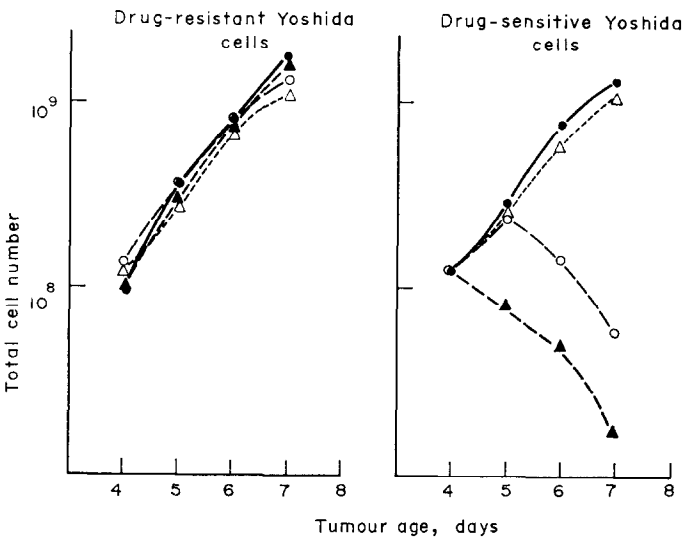

FIG. 1. Growth curves of sensitive and resistant Yoshida ascites cells in tumour-bearing animals following drug treatment. ●——●, Solvent treated; △- - -△, phenobarbitone treated; ○- - -○, chlorambucil treated; ▲- - -▲, phenobarbitone + chlorambucil treated. Each point represents the mean from four animals.

of cells were unaffected by treatment with phenobarbitone alone. The proliferation of resistant cells was not altered either by chlorambucil alone or by the combination of phenobarbitone and chlorambucil. However, in the animals bearing the drug-sensitive tumour this combination produced a greater cell kill than in those treated with

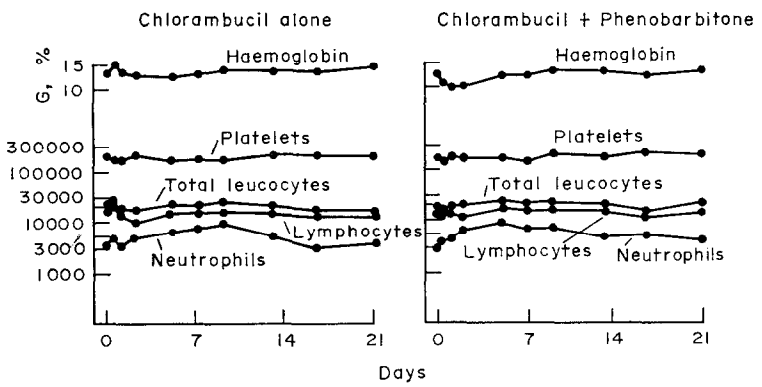

FIG. 2. Peripheral blood chart of rats treated with chlorambucil alone or with a combination of phenobarbitone and chlorambucil. Each point represents the mean from four animals.

chlorambucil alone. This drug schedule caused no greater alteration in the peripheral blood picture than did the administration of chlorambucil alone (Fig. 2).

The variation in content of DNA, RNA and protein in sensitive and resistant tumour cells after treatment is shown in Table 3. No significant change occurred in

TABLE 3. LEVELS OF DNA, RNA AND PROTEIN IN SENSITIVE AND RESISTANT YOSHIDA ASCITES CELLS FOLLOWING DRUG TREATMENT OF TUMOUR-BEARING ANIMALS

Cells	Drug treatment *in vivo*	Days after drug treatment	DNA ($mg/10_9$ cells)	RNA ($mg/10_9$ cells)	Protein ($mg/10_9$ cells)
Resistant	None		14·7	36·0	102
Resistant	Phenobarbitone	1	15·0	40·2	113
	only	2	15·2	36·3	107
Resistant	Chlorambucil	1	15·8	38·2	97
	only	2	15·2	39·0	112
Rcsistant	Phenobarbitone +	1	15·4	32·6	112
	chlorambucil	2	15·2	37·0	106
Sensitive	None		15·1	37·0	108
Sensitive	Phenobarbitone	1	15·6	40·4	154
	only	2	15·0	39·0	116
Sensitive	Chlorambucil	1	22·0	40·4	250
	only	2	24·0	59·7	406
Sensitive	Phenobarbitone +	1	20·2	53·1	365
	chlorambucil	2	25·0	74·4	821

The growth curves of ascites cells in tumour-bearing animals following drug treatment are shown in Fig. 1. Each value represents the mean of eight determinations. The standard error for DNA determinations ± 0·3, for RNA determinations ± 1·0 and for protein estimations ± 6 per cent.

any of these properties in the resistant cells. The sensitive cells showed a 50 per cent increase in protein content 24 hr after phenobarbitone treatment but the level had returned to control values 24 hr later. The previously reported increase in the levels of all these parameters in the sensitive cells after chlorambucil treatment was confirmed.[4] These increased levels were further enhanced when a combination of chlorambucil and phenobarbitone was given, with the cellular protein content having trebled 24 hr after chlorambucil treatment, whilst by 48 hr, the value had increased approx. 8-fold.

3-Methylcholanthrene and Sudan III also enhance the toxic effects of chlorambucil in drug-sensitive cells but the results are not reproduced here, since the growth curves are identical to those shown in Fig. 1.

Studies in vitro. The concentrations of enzyme inducers used (Table 1) were without effect on the growth rates of the various cell lines. Protein synthesis was followed over a 12-hr period after the addition of phenobarbitone to the culture medium. The results, shown in Table 4, indicate that, by 12 hr, the rate of protein synthesis, expressed as counts/min of ^3H incorporated per milligram of protein, had increased in both drug-sensitive Yoshida cells and L5178Y cells to 150 per cent of control values. Under no conditions was any increase observed in the rate of protein synthesis in drug-resistant Yoshida cells.

The effects *in vitro* of pretreatment with enzyme inducers on the LD_{90} of chlorambucil (that dose required to reduce the surviving fraction of cells to less than 10 per

TABLE 4. EFFECTS OF PHENOBARBITONE TREATMENT (2 mM) ON THE RATE OF PROTEIN SYNTHESIS IN TUMOUR CELLS GROWN *in vitro*

Cell line	Treatment *in vitro*	Counts/min per mg protein*
Sensitive Yoshida	None	$2{\cdot}13 \times 10^6$
Sensitive Yoshida	Phenobarbitone	$3{\cdot}23 \times 10^6$
Resistant Yoshida	None	$2{\cdot}28 \times 10^6$
Resistant Yoshida	Phenobarbitone	$2{\cdot}19 \times 10^6$
L5178Y	None	$2{\cdot}25 \times 10^6$
L5178Y	Phenobarbitone	$3{\cdot}45 \times 10^6$

* Results in this table are expressed as counts/min of tritium incorporated per milligram of protein. Each point represents the mean of eight determinations. The overall scatter about any point ≯ 5 per cent.

cent) are shown in Table 5. For each inducer, the LD_{90} is decreased by a factor 2–4 in drug-sensitive Yoshida and L5178Y cells. This treatment schedule had no effect on the response of drug-resistant Yoshida cells to chlorambucil treatment.

TABLE 5. ALTERATIONS OF THE LD_{90} OF CHLORAMBUCIL FOR LINES OF TUMOUR CELLS GROWN IN SUSPENSION CULTURE FOLLOWING PRETREATMENT WITH PHENOBARBITONE (2 mM)

Cell line	LD_{90} Chlorambucil alone	*LD_{90} Chlorambucil after phenobarbitone pretreatment
Sensitive Yoshida	$3{\cdot}3 \times 10^{-6}$ M	10^{-7} M
L5178Y	5×10^{-5} M	2×10^{-5} M
Resistant Yoshida	10^{-4} M	10^{-4} M

* Identical values for LD_{90} of chlorambucil were obtained in similar experiments following pretreatment with 3-methylcholanthrene (0·1 mM) or benz[a]anthracene (0·1 mM). Each value represents the mean of at least eight determinations. The overall scatter about any point ≯ 10 per cent.

DISCUSSION

These results indicate that the toxicity of chlorambucil to alkylating agent-sensitive tumour cells growing both *in vivo* and *in vitro* can be enhanced by prior administration of enzyme inducers. This effect has been demonstrated using a range of chemical compounds which are known to share the property of inducing the synthesis of microsomal drug-metabolizing enzymes.[3] This effect *in vivo* appears to be selective against tumour cells: the response patterns of the peripheral blood in the intact animal are similar whether chlorambucil is given alone or following phenobarbitone, implying that, after enzyme induction, there is no concomitant increase in the number of cells killed in the normal marrow. This drug combination also eliminates the ability of drug-sensitive Yoshida cells to degrade chlorambucil *in vitro*.

None of these events have been shown to occur in alkylating agent-resistant Yoshida cells. Other workers have reported that almost all established mammalian cell lines in

culture lack basal or inducible levels of microsomal drug-metabolizing enzymes (e.g. aryl hydrocarbon hydroxylase):[13] two exceptions are the induction of the hydroxylase in m3T3 and HeLa cell cultures.[14] Furthermore, Donelli *et al.*[15] failed to demonstrate that the microsomal fractions from a series of transplantable tumours were able to metabolize foreign compounds such as *p*-nitroanisol and aniline. It has also been observed that transformed cells, in contrast to normal cells, lack the ability to metabolize hydrocarbons.[16] It may, therefore, be significant that the failure of resistant cells to enter "unbalanced growth" in response to alkylating agents[4,5] is reflected here in their inability to respond to microsomal enzyme inducers.

Cyclophosphamide, an alkylating agent which is known to require microsomal metabolism before it can exert cytotoxic effects, has been shown to cause a greater tumour-cell kill in tumour-bearing rats which had been pretreated with phenobarbitone.[17]

Although chlorambucil is generally thought to be a direct acting alkylating agent,[18] these results indicate that metabolic activation may also be involved in the antitumour activity of this compound in drug-sensitive cells.

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