Biochemical Pharmacology, Vol. 19, pp. 1387–1397. Pergamon Press. 1970. Printed in Great Britain

STUDIES ON THE MECHANISM OF RESISTANCE TO ALKYLATING AGENTS OF THREE ASCITES TUMOURS IN THE RAT

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(*Received* 1 *July* 1969; *accepted* 18 *October* 1969)

Abstract—Three rat ascites tumours, namely the Yoshida ascites sarcoma, a line developed from it resistant to alkylating agents, and the resistant Novikoff ascites hepatoma, were compared in an attempt to correlate sensitivity to alkylating agents with certain biochemical and physiological properties. The tumours did not differ significantly in growth rates, although the number of cells required to produce a tumour was higher for the Novikoff tumour than for the Yoshida tumours. No differences were found between the two Yoshida tumours in cellular content of DNA, RNA, free nucleotides and total cell protein, though in the Novikoff tumour there was a higher cell content of total cell protein than in the Yoshida tumours. There was, however, a lower level of protein-free thiol in the Yoshida sensitive tumour than in the Yoshida resistant tumour and an even lower level in the Novikoff tumour. *In vivo* incorporation of radioactivity (after administration of ^{3}H-Melphalan) by the Yoshida resistant and sensitive tumours at peak level was three times that of the Novikoff tumour. *In vivo* incorporation of ^{3}H-Aniline mustard into the Novikoff cell, though the same as that of the Yoshida sensitive tumour at peak level (30 hr) occurred more slowly. Preliminary studies on the binding of ^{3}H-Aniline mustard to DNA at 30 hr after injection indicate approximately the same binding in all three tumours. Possible mechanisms of resistance to alkylating agents of the Yoshida resistant and Novikoff cells are discussed.

IN VIEW of limited existing knowledge as to why certain tumours are resistant to alkylating agents and others are sensitive, an attempt has been made to explain this phenomenon by comparing the properties of three ascites tumours selected on the basis of their variation in sensitivity. The tumours were compared for growth rates, the number of cells required to be injected in order to establish reliable tumour growth, their response to several doses of alkylating agents *in vivo* (and in the case of Melphalan, *in vitro* as well), response to several doses of two antimetabolite drugs *in vivo*, for cellular content of DNA, RNA, free nucleotides, total cell protein and protein-free thiol, and for their ability to chemically react with ^{3}H-Melphalan and ^{3}H-Aniline mustard. Preliminary studies on the binding of ^{3}H-Aniline mustard to the DNA of these cells *in vivo* were also performed.

MATERIALS AND METHODS

The tumour cell lines used were all in the ascites form. They were respectively, the Yoshida ascites sarcoma, a line derived from it considerably more resistant to

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alkylating agents,[1] and the Novikoff ascites hepatoma, which is naturally resistant to alkylating agents. Tumours were passaged by withdrawal of 0·5 ml of 3- to 5-day-old ascites from the donor and by aseptic intraperitoneal transference into rats of the pure line inbred Wistar Albino Glaxo variety, weighing between 30 g and 50 g. Both sexes were used, though only one for any particular experiment. They were fed on Dixon's diet 86 and water *ad lib.* All three tumours were obtained from Dr. T. A. Connors of the Chester Beatty Institute. Benzylpenicillin and streptomycin were added to the ascitic fluid immediately prior to injection.

Growth studies were performed by counting aliquots of the total volume of ascites cells on a haemocytometer at intervals after injection intraperitoneally, of a fixed number of cells.

Tumour dose–response *in vivo* was measured for each tumour independently by injecting i.p. 0·2-ml aliquots of fresh ascites cell suspension (10^7 cells/ml) into groups of five weanling rats on day 0. On day 5 each group received a dose of drug given s.c. into the scruff of the neck. The animals were weighed on day 0 and at regular intervals thereafter. Survival times of the animals were noted and used as a measure of drug effect.

Melphalan (*p*-di-2-chloroethylamino-L-phenylalanine) was dissolved in absolute ethanol containing 2% v/v HCl and this was then added to 9 vol. propylene glycol buffer (2% w/v K_2HPO_4; 4·5% v/v propane 1:2 diol, in water), immediately prior to injection. Methotrexate (American Cyanamid Co.) was given dissolved in water, 6-Mercaptopurine (Burroughs Wellcome Co.) was ultrasonicated in arachis oil and given as an oily suspension and aniline mustard (*N*-*N*-di-2-chloroethylaniline) was given in dimethylsulphoxide. Control groups of rats received the respective solvents.

Tumour dose–response *in vitro* was estimated by removing fresh ascites tumour cells from the peritoneal cavity and incubating aliquots *in vitro* with different concentrations of Melphalan, essentially by the method described by Ball, Connors, Double, Ujhazy and Whisson.[2] Drug was added in 0·1 ml of solvent and after incubation, aliquots of suspension containing 10^7 tumour cells were injected into groups of fresh hosts.

Biochemical studies. Samples of ascitic tumour were harvested from tumour-bearing animals 4–6 days after passaging. The cells were then resuspended in 1:2 saline:distilled water (to lyse the red blood cells present) and after standing for 5 min, they were centrifuged and the supernatant discarded. An aliquot of resuspended tumour cells was then counted as previously described. The remaining suspension was then centrifuged and the precipitate treated by a modification of the method of Schneider, Hogeboom and Ross.[3] The precipitate was treated with 5·0 ml of 0·2 N perchloric acid at 0°. After mixing and centrifuging for 3 min the supernatant was decanted and retained. This procedure was repeated with a further 5·0-ml aliquot of 0·2 N perchloric acid and the two supernatants combined. The supernatant was then used to assay free nucleotides. The precipitate was then treated with 5·0 ml of absolute ethanol and after mixing and centrifuging, the supernatant was discarded. The procedure was repeated using a 5·0-ml aliquot of 3:1 ether:ethanol, the supernatant being discarded. The precipitate was then treated with 5·0 ml of 0·5 N perchloric acid at 70° for 20 min. After centrifuging the supernatant was retained, and the procedure repeated with another 5·0 ml of perchloric acid, the two supernatants then being combined and used for estimation of RNA and DNA. The remaining precipitate was dissolved in 10 ml

of 0·1 N NaOH and allowed to stand for 24 hr, after which it was used to measure total cell protein.

The fractions were assayed for free nucleotides by spectrophotometric analysis at 260 mμ, for DNA by the diphenylamine method of Burton[4] (both using Salmon testes DNA as standard: British Drug Houses), for RNA by the orcinol method of Brown[5] (using yeast RNA, Sigma Chemicals, as standard) and for protein by the Folin reagent method of Lowry *et al.*,[6] using bovine serum albumin as standard.

Non-protein bound thiol was measured spectrophotometrically by the method of Ellmann,[7] having been extracted as previously described by Connors *et al.*[8]

Studies with radioactive drugs. Individual weanling rats were injected with approximately 5×10^7 tumour cells. Three animals were used per time point per tumour. On day 4 after tumour injection, rats were injected subcutaneously with 2 mg/kg of ^{3}H-Melphalan.*

Animals were sacrificed at intervals after injection of ^{3}H-Melphalan and portions of the tumour and liver removed. The tumour was then washed in 1:2 saline:distilled water (to break up and lyse red blood cells). After centrifuging and decanting off the supernatant, the cell precipitate was broken up by adding 2·5 ml of distilled water per gram of tissue and shaking. Of this tumour cell lysate, 1·0 ml was pipetted into a weighed dish and heated overnight at 60° to dry weight. 0·5 ml was dissolved in 1·0 ml of TEH (tetraethylammonium hydroxide) and was then used for the estimation of total cell uptake of Melphalan. In cases where the bound content was estimated, 0·5 ml of the cell suspension was added to 1·0 ml of 10% w/v SSA (sulphosalicylic acid) at 0° and after mixing was centrifuged hard. The supernatant was decanted off and was used to measure the "free" Melphalan in the cell (i.e. that not bound to macromolecules). The precipitate was dissolved in 0·5 ml of TEH and was used to measure the bound Melphalan in the cells. In all cases a weighed portion of liver from the tumour-bearing animal was dissolved in TEH (1·0 ml of TEH/300 mg of liver) and counted for radioactivity. All the TEH solutions were heated at 70° until they were homogenous. Aliquots of 0·1 ml of these solutions (and the SSA solutions) were added to 10 ml of a dioxane–ethanol scintillation counting fluid and were counted on a Packard Tricarb Scintillation counter.

Essentially the same procedure was employed for ^{3}H-Aniline mustard with the following modification. A dose of 20 mg/kg ^{3}H-Aniline mustard (specific activity 80 mc/mM, as prepared by Ball and Wade[9]) was administered in dimethylsulphoxide.

Preliminary binding studies of ^{3}H-Aniline mustard to DNA in vivo. Rats bearing tumours were injected subcutaneously with 20 mg/kg of ^{3}H-Aniline mustard (specific activity 350 mc/mM and obtained from Dr. C. R. Ball) dissolved in dimethylsulphoxide. At intervals of 4 and 30 hr after injection of drug, samples of tumour were removed from the host and the DNA extracted by a method based on those of Kirby.[10, 11]

Statistically significant differences are stated as being so when $P < 0{\cdot}05$.

RESULTS

Growth curves of the three tumours are shown in Fig. 1. There is no significant difference between growth rates of the tumours. The relationship between the number of tumour cells injected into the peritoneal cavity and the survival time of the hosts is

* As prepared by R. Wade and T. S. Murthy, unpublished work.

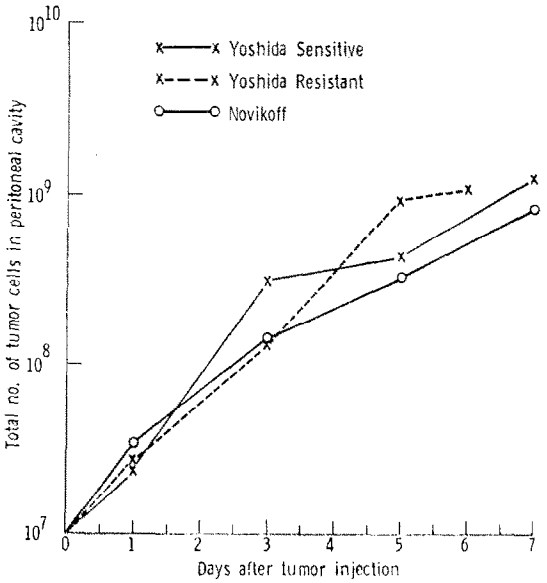

FIG. 1. Growth curves of three ascites tumours in the rat.

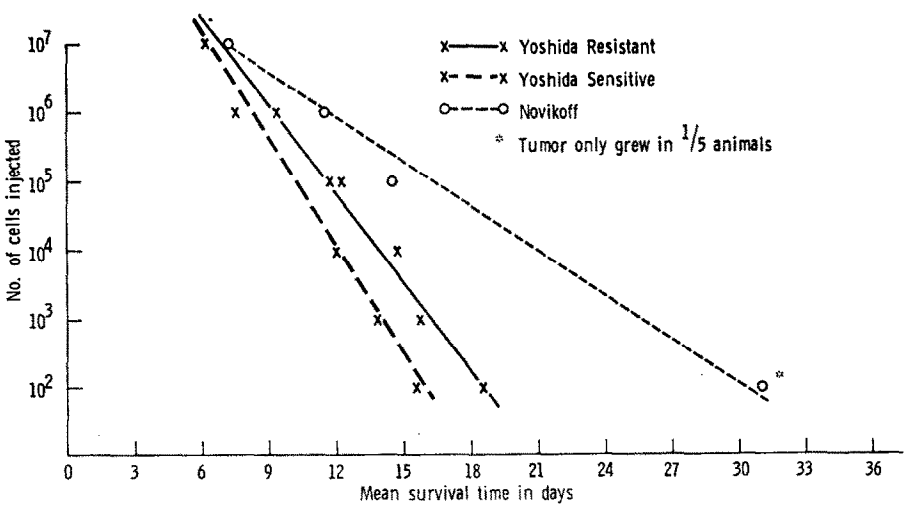

FIG. 2. Relationship between survival time and number of tumour cells injected into the peritoneal cavity of rats.

shown in Fig. 2. In contrast to the results of Ball *et al.*[2] who used the Chester Beatty rat as host, and found difficulty in obtaining reliable tumour growth with an inoculation of less than 10^5 cells of either of the two Yoshida tumours, these two tumours grew reliably (i.e. 80% tumour "takes" or more) in the Glaxo rat, from as few as 100 cells. As can be seen, there is a good linear relationship between $\log_{10}$ number of cells injected and survival time of the host. If survival time is to be used as a method for estimating the effect of a dose of drug, this linear relationship is required to be

established before an estimate of percentage tumour cells killed by the drug can be made. A linear relationship was also found for the Novikoff tumour, though this was only so with injections of 10^5 cells or more. This tumour did not grow when fewer than 10^5 cells were injected (except for one isolated case).

The responses of tumour-bearing animals to the alkylating agent, Melphalan, are shown in Table 1. All animals given doses of Melphalan of 16·2 mg/kg or more died

TABLE 1. EFFECT OF MELPHALAN ON SURVIVAL TIME OF RATS BEARING ASCITES TUMOURS

	Yoshida-sensitive		Yoshida-resistant		Novikoff	
Dose of Melphalan (mg/kg, s.c.)	No. of animals dying of tumour	Mean survival time (days)	No. of animals dying of tumour	Mean survival time (days)	No. of animals dying of tumour	Mean survival time (days)
Control	5/5	9·4	5/5	9·4	5/5	8·8
0·2	5/5	14·0	5/5	10·4	5/5	9·3
0·6	5/5	20·2	5/5	8·6	5/5	11·8
1·8	2/5	28·0*	5/5	11·0	5/5	9·0
5·4	1/5	20·0	5/5	12·2	5/5	9·0
16·2	0/5	8·8†	0/5	9·6†	5/5	10·4‡
48·6	0/5	8·0†	—	—	—	—

In all cases the drug was administered as a single dose 5 days after injection of tumour.
* Mean survival time of animals which died within 60 days from effects of the tumour.
† Animals dying due to drug toxicity with no tumour present.
‡ Animals dying due to drug toxicity with tumour present.

due to drug toxicity. The LD_{50} was estimated to be approximately 10 mg/kg. All animals given these high doses suffered heavy weight loss. Both the Yoshida sensitive and Yoshida resistant tumours were destroyed by lethal doses of Melphalan, i.e. in killing the animal, the drug also killed the tumour. Animals bearing the Novikoff tumour, however, although killed by drug toxicity at this dose, still possessed a large ascites tumour at death. This would indicate that even a dose of Melphalan toxic to the host would not kill the tumour *in vivo*. This suggests that *in vivo* at least, the Novikoff tumour is more resistant to Melphalan than the Yoshida resistant tumour. At sublethal doses of Melphalan the survival times of animals bearing the Yoshida sensitive tumour were significantly extended by doses of Melphalan as low as 0·2 mg/kg (2% of the LD_{50} dose) and with doses of 1·8 mg/kg and 5·4 mg/kg some animals survived longer than 60 days and were defined as apparent cures. No significant extensions of survival time were noted with any sublethal doses of Melphalan given to animals bearing the Yoshida resistant or Novikoff tumours. Hence the order of sensitivities to Melphalan in this system *in vivo* would appear to be Yoshida sensitive > Yoshida resistant > Novikoff.

Table 2 shows the effect of aniline mustard on animals bearing the Yoshida sensitive and Novikoff tumours respectively. No apparent cures of animals bearing Yoshida sensitive tumours were observed, though significant extensions of survival time were noted with doses of 54 mg/kg and 162 mg/kg. In this host the LD_{50} of aniline mustard was found to be approximately 600 mg/kg. No significant extensions of survival time were observed with animals bearing the Novikoff tumour in doses of aniline mustard up to 486 mg/kg. Hence a similar spectrum of activity was observed against these two

TABLE 2. EFFECT OF ANILINE MUSTARD ON SURVIVAL TIME OF RATS WITH ASCITES TUMOURS*

Dose of Aniline mustard (mg/kg, s.c.)	Yoshida-sensitive		Novikoff	
	No. of animals dying of tumour	Mean survival time (days)	No. of animals dying of tumour	Mean survival time (days)
Control	5/5	8·4	5/5	10·0
2	—	—	5/5	10·4
6	4/4	9·3	4/4	10·3
18	5/5	11·8	5/5	12·0
54	5/5	15·2	5/5	12·2
162	5/5	19·0	5/5	13·8
486	5/5	16·8	—	—

* In all cases the drug was administered as a single dose 5 days after injection of the tumour.

tumours with Aniline mustard as was seen with Melphalan. The anti-metabolite drugs methotrexate and 6-mercaptopurine caused no significant extensions of survival times in animals bearing any of the three tumours used with doses up to 24·3 mg/kg and 810 mg/kg respectively.

Approximate doses of Melphalan were found for all three tumours, which after incubation with tumour cells *in vitro* at 37° for 2 hr (10^7 cells of which were then reinjected into fresh hosts), caused minimum significant extensions of survival time of hosts over controls. The doses found were 0·01–0·02 μg/ml, 1·0 μg/ml and 0·5 μg/ml of Melphalan for the Yoshida sensitive, Yoshida resistant and Novikoff tumours respectively. In the case of the Novikoff tumours, a dose of 0·2 μg/ml gave rise to no significant extension of survival time of hosts, whilst doses of 0·5 μg/ml and above gave rise to apparently tumour-free animals on reinjection of the tumour cells, since the animals survived longer than 60 days, well in excess of the time theoretically required for one surviving tumour cell to proliferate to a lethal number. Hence *in vitro*, it would appear that the order of sensitivity to Melphalan was Yoshida sensitive > Novikoff > Yoshida resistant. This differs from the *in vivo* case, where the apparent order of sensitivities was Yoshida sensitive > Yoshida resistant > Novikoff.

The cellular contents of RNA, DNA, free nucleotides, protein and protein-free thiol of the three tumours used are shown in Table 3. The free nucleotide content per gram of protein is greater in the two Yoshida tumours than in the Novikoff tumour.

TABLE 3. CELLULAR COMPONENTS OF ASCITES TUMOUR CELLS

Cell constituent	Novikoff		Yoshida sensitive		Yoshida resistant	
	Mean	S.D.	Mean	S.D.	Mean	S.D.
DNA*	86·3	14·5	78·9	7·1	81·1	18·5
RNA*	156·0	19·8	163·0	16·6	160·0	13·7
Free nucleotides*	19·7	4·4	30·2	10·3	30·7	7·4
Protein†	28·0	6·2	15·3	3·2	14·5	1·6
Protein-free thiol‡	2·86	0·67	6·40	1·37	10·5	1·48

* mg/g of protein.
† g/cell × 10^{11}.
‡ μg SH/100 mg protein.

The absolute quantity of protein in the Novikoff cell is greater than in the two Yoshida tumours, which was expected since the Novikoff tumour cell is larger in volume. All three tumours were significantly different from one another in their protein-free thiol content. This was the only parameter of those investigated biochemically, in which the Yoshida sensitive and Yoshida resistant tumours were found to differ. The protein-free thiol content of the Yoshida resistant tumour was approximately 60 per cent higher than that of the Yoshida sensitive and 4 times that of the Novikoff cell.

The relative degrees of chemical reactivity (as measured by uptake of label) by the three tumours *in vivo* after injection of ^{3}H-Melphalan (2 mg/kg) are shown in Fig. 3.

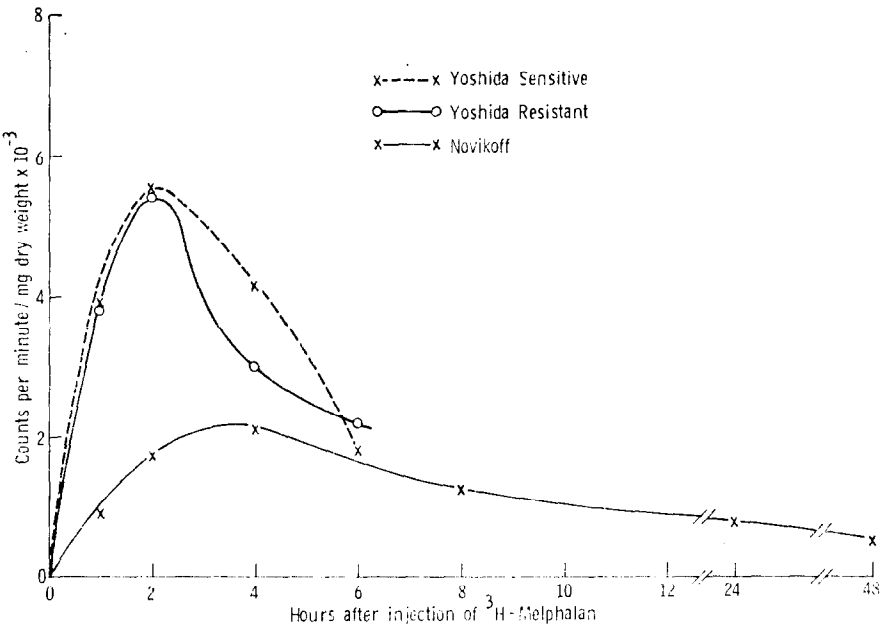

Fig. 3. Uptake of ^{3}H-Melphalan (2 mg/kg) *in vivo* by ascites tumour cells.

There was no significant difference between the amounts reacting with the Yoshida sensitive and Yoshida resistant cells as measured, though the uptake by the Novikoff tumour, however, was significantly lower. At 2 hr after Melphalan injection there was three to four times as much Melphalan present in the two Yoshida tumours as there was in the Novikoff tumour. It is worth noting that the peak time of uptake of drug by the Novikoff was 4 hr after drug as compared with 2 hr in the case of the two Yoshida tumours as is shown in Fig. 3, indicating a slower rate of reaction with the Novikoff tumour.

At this time the degree of binding of ^{3}H-Melphalan to intracellular macromolecules was also measured. The results shown in Table 4 are the mean of four independent determinations. As in the case of the total cell uptake of ^{3}H-Melphalan, there was no difference in binding between the two Yoshida tumours, but both bound ^{3}H-Melphalan to a higher degree than the Novikoff tumour as shown in Table 4.

In vivo reaction of ^{3}H-aniline mustard with the Yoshida sensitive and Novikoff

TABLE 4. RADIOACTIVITY BOUND TO MACROMOLECULES OF TUMOUR CELLS 2 hr AFTER ^{3}H-MELPHALAN 2 mg/kg

Tumour	c.p.m./mg dry weight*
Novikoff	84
Yoshida resistant	156
Yoshida sensitive	170

* Mean of four determinations.

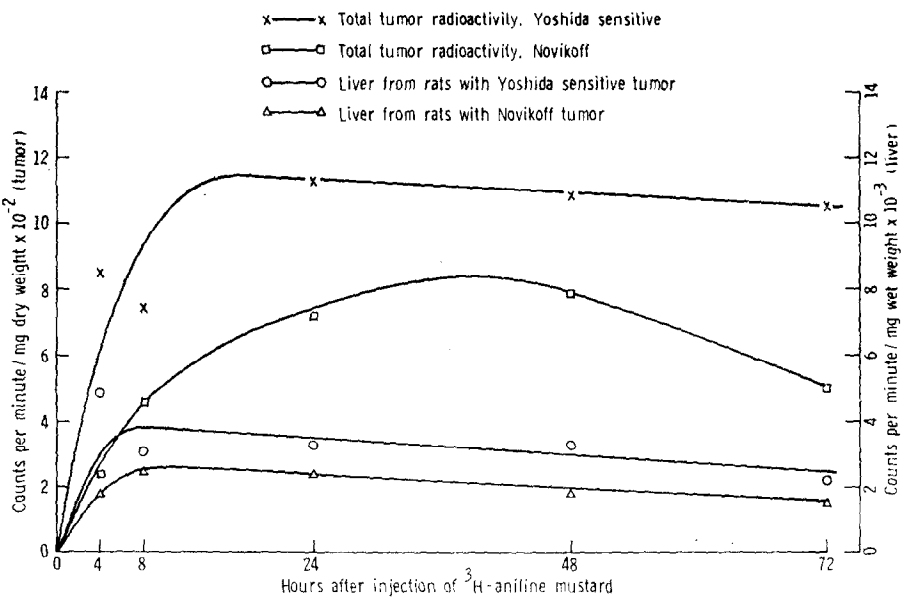

FIG. 4. Uptake of ^{3}H-Aniline mustard (20 mg/kg) by ascites tumour cells and livers of tumour bearing animals.

TABLE 5. RADIOACTIVITY BOUND TO DNA OF TUMOUR CELLS 30 hr AFTER ANILINE MUSTARD 20 mg/kg

Tumour	c.p.m./mg of DNA*
Novikoff	148
Yoshida resistant	112
Yoshida sensitive	126

* Mean of two determinations.

tumours is shown in Fig. 4. As can be seen, the difference, if any, between the highest points of uptake is small, though the rate of uptake is possibly more rapid in the Yoshida sensitive tumour and liver in the early stages than in the Novikoff tumour. The apparent drop in labelling of the Novikoff cell at 72 hr after ^{3}H-Aniline mustard is probably due to "dilution" of the label by continued nucleic acid synthesis, since this cell is more resistant to the drug than the Yoshida sensitive cell. The dose used,

20 mg/kg, was less effective in instigating extensions of survival time in animals bearing Yoshida sensitive cells than was 2 mg/kg of Melphalan and this is worth considering on examination of the chemical reaction patterns for these two tumours.

Preliminary results using ^{3}H-Aniline mustard of high specific activity to measure binding to DNA indicated, as is shown in Table 5, that at 30 hr after injection of label (a time at which total cell reaction with drug was approximately the same in the Yoshida sensitive and Novikoff tumours) binding of the drug to DNA was approximately the same for all three tumours. There was no detectable binding of this drug to the DNA of any of these cells at 4 hr after drug injection.

DISCUSSION

An attempt has been made to correlate some physical or biochemical property of three ascites tumour cell lines with their varying sensitivities to alkylating agents. No significant difference could be found between the growth rates (as measured by the total cell population at fixed times after a known number of cells were inoculated).

The Yoshida sensitive and resistant cells appeared to be very prolific, developing into lethal ascites tumours from as few as 100 cells, whilst the Novikoff tumour did not grow reliably from less than 10^5 cells. As Skipper[12] has pointed out, the fewer cells required to establish a tumour, the more difficult it is to permanently inhibit the tumour, since the percentage of tumour cells needed to be killed in order to effect a "cure" will be higher. Thus in the case of the Novikoff tumour, the effectiveness of drug therapy might be anticipated since this tumour is normally reluctant to grow from less than 10^5 cells. This, however, is apparently not so indicating the very high degree of cellular resistance of this tumour to these drugs.

Biochemically, the major feature of interest which might indicate the nature of resistance in these tumours, is their protein-free thiol content. The figures for the Yoshida sensitive and Yoshida resistant tumours agree well with those already reported for the solid Yoshida sarcomata by Hirono[13] and Ball *et al.*[2] Thiols have been shown to combine with alkylating agents *in vitro* and *in vivo* by Roberts and Warwick[14-16] and much work as to the significance of thiols reacting with alkylating agents has been published (Calcutt and Connors,[17] Connors, Jeney and Jones,[18] Ball and Connors[19]). Since this reaction with alkylating agents by thiols renders the former pharmacologically inactive and hence prevents the drug from reaching the critical site of antitumour action in an active form, the excess thiol in the Yoshida resistant tumour may, by inactivating the alkylating agent, account for this cell's resistance to such drugs. It seems likely, however, that the excess thiol present in the Yoshida resistant tumour has to be present either as a different type or if the same type, in a different form from that in the Yoshida tumour, since if this is to account directly for why the Yoshida resistant tumour is resistant, a 60 per cent increase in thiol has to explain a greater than 27-fold increase in tumour cell tolerance to Melphalan *in vivo* and a 50-fold increase *in vitro*. If a high intracellular protein-free thiol content can account for the resistance of the Yoshida resistant tumour to alkylating agents, it is unlikely that the same applies to the Novikoff tumour since its protein-free thiol content is much lower than either of the Yoshida tumours discussed.

As was described in the Results section, the order of sensitivities of the tumours to Melphalan *in vivo* was Yoshida sensitive > Yoshida resistant > Novikoff. However, the results of the dose–response relationship *in vitro*, would suggest that *in vitro* the

order of sensitivities was Yoshida sensitive > Novikoff > Yoshida resistant. A possible explanation of the apparent discrepancy between the *in vivo* and *in vitro* results can be postulated on consideration of the different conditions of drug/tumour interaction. In the *in vivo* case the level of Melphalan in the blood (and as a result, it is assumed, the concentration in contact with the tumour) rise and fall relatively rapidly after drug administration. This would mean that a tumour cell capable of reacting with a drug rapidly would absorb more before the greater portion of the drug had been removed from the blood, than would a tumour which absorbs drug slowly. In the *in vitro* case, however, the concentration of Melphalan in contact with the tumour remains virtually the same for 2 hr (this is not strictly true since some Melphalan will bind to proteins in the culture medium during incubation and some will hydrolyze to the inactive hydroxy-form of Melphalan, both of which events also occur, however, in the *in vivo* case). This period gives a tumour with a slower rate of reaction time to absorb drug to a maximum level, which in absolute terms may be higher than that for the tumour cell which absorbs drug more rapidly. As indeed seemed likely from a comparison of these *in vivo* and *in vitro* responses to Melphalan, studies using ^{3}H-Melphalan and ^{3}H-Aniline mustard demonstrate a slower rate of reaction of drug by the Novikoff tumour. It would seem that in the Novikoff tumour resistance to Melphalan could be accounted for by the simple fact that less drug reacts with the cell. However, consideration of the maximum chemical reaction of ^{3}H-Aniline mustard by the two tumours (Yoshida sensitive and Novikoff) and the preliminary results of binding of this drug to DNA, indicates that there is no great difference between the two tumours.

If, as Wheeler[20] has concluded, DNA is the active site of antitumour and/or cytotoxic action, then removal of alkylated sections and subsequent repair of the DNA would seem to be a likely mechanism of resistance to alkylating agents in the Novikoff cell. Roberts, Crathorn and Brent[21] have recently demonstrated the likelihood of repair of DNA in HeLa cells subjected to alkylation with sulphur mustard.

Acknowledgements—The author wishes to express his thanks to his supervisors, Professor E. C. Amoroso and Dr. T. A. Connors for their continual help and advice, to Mr. John Boyd for technical assistance, and to Dr. F. S. Philips for his advice in preparing this manuscript. This work constitutes part of a thesis submitted for the degree of Ph.D. in the University of London, with a grant provided by the Medical Research Council.

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