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THE EFFECTS OF CHLORAMBUCIL ON THE UTILIZATION OF EXOGENOUS THYMIDINE BY TUMOUR CELLS

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Abstract—Treatment of an alkylating agent-sensitive strain of the Yoshida ascites sarcoma with chlorambucil resulted in an inhibition of the incorporation of [^{3}H]thymidine into DNA, which could be overcome by incubating cells in high extracellular concentrations of thymidine. Increase in cellular DNA content and the dilution of specific radioactivity in pre-labelled DNA indicated that DNA synthesis was continuing at times when [^{3}H]thymidine incorporation was inhibited. Uptake and phosphorylation of thymidine were not impaired by the treatment and the reduced incorporation of [^{3}H]thymidine into DNA is attributable to a block in the utilization of TTP derived from exogenous nucleoside.

An early inhibition of incorporation of [^{3}H]thymidine into DNA is often observed following treatment of tumour cells with alkylating agents (reviews by Wheeler [1, 2]). However, there is much evidence to support the hypothesis that alkylating agents arrest cells in the G2 phase of the cell cycle [3–7]. Mitosis would seem to be more sensitive to the effects of alkylating agents than DNA synthesis [7, 8], an inhibition of incorporation of [^{3}H]thymidine into DNA being a result, not a cause, of failure to enter mitosis [4]. At higher doses of alkylating agents DNA synthesis itself may be immediately affected.

A consequence of inhibition of mitosis is that the average DNA content per cell doubles [5]. This is the situation when Yoshida ascites sarcoma cells were treated *in vivo* with chlorambucil at a dose of 8 mg/kg [9]. It has also been reported that under these conditions there was no inhibition of incorporation of [^{3}H]thymidine into DNA [10] in the first 24 hr after treatment, compatible with the increase in DNA content per cell. These experiments, however, were done using high extracellular concentrations of [^{3}H]thymidine ($2{\cdot}5 \times 10^{-3}$ M). As described in the present paper, when experiments were repeated using much lower extracellular concentrations of labelled nucleoside (4×10^{-8} M) an inhibition of incorporation into DNA was observed at times when DNA synthesis was continuing. We have attempted to explain (a) the early inhibition of incorporation of exogenous [^{3}H]thymidine into DNA and (b) the reversal of this inhibition by high extracellular concentrations of thymidine. We have, therefore, examined the uptake of [^{3}H]thymidine and its conversion to phosphorylated derivatives under the two sets of conditions.

MATERIALS AND METHODS

Chemicals. Chlorambucil (Leukeran), $(ClCH_2CH_2)_2.N.C_6H_4.(CH_2)_3COOH$, was synthesized in the Chester Beatty Research Institute. Inulin [^{14}C]carboxylic acid (sp. act. 8·6 mCi/m-mole) and [^{3}H]thymidine (25 Ci/m-mole) were obtained from the Radiochemical Centre, Amersham, U.K. Thymidine was obtained from Sigma Chemical Company, Surrey, U.K. Other chemicals were purchased from either Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K. or British Drug Houses Ltd., Poole, Dorset, U.K., AnalaR grades being used where available.

Animal experimentation. Full details of animal experimentation and tumour transplantation techniques have been provided previously [9, 11]. Chlorambucil was administered subcutaneously to tumour-bearing animals at a dose of 8 mg/kg body wt on the 4th day following tumour transplantation.

[^{3}H]*Thymidine incorporation studies.* Animals were killed at various times after drug treatment, the ascitic fluid aspirated with ice-cold isotonic saline and the tumour cells harvested by centrifugation at 600 *g* at 4° for 10 min. The cells were then incubated (10^7 cells/ml) at 37° for 30 min in Fischer's tissue culture medium containing either $2{\cdot}5 \times 10^{-3}$ M [^{3}H]thymidine (4 mCi/m-mole) or 4×10^{-8} M [^{3}H]thymidine (25 Ci/m-mole). After incubation, the cells were again centrifuged at 600 *g* at 4° for 10 min, the supernatant discarded and the cell pellet washed with two 1-ml aliquots of ice-cold 0·2 N perchloric acid (PCA) which were subsequently pooled for liquid scintillation counting in an Intertechnique Model SL40 liquid scintillation counter: 0·2-ml aliquots were added to 10 ml of scintillation fluid. The remaining pellet was then hydrolysed at 70° for 20 min with two 1-ml aliquots of 0·5 N perchloric acid which were pooled for liquid scintillation counting (0·2 + 10 ml scintillation fluid). DNA was estimated by the method of Burton [12].

Determination of the volume of cell water in ascites cells. At various times after treatment cells were harvested as described above. An aliquot of cell suspension containing 10^7 cells was centrifuged at 600 *g*, 4° for 10 min and resuspended in 1 ml of Fischer's medium containing inulin [^{14}C]carboxylic acid (0·25 μCi/ml). After 5 min at room temperature the suspensions were spun at 600 *g*, 4° for 10 min. The supernatant was poured off and 0·1-ml aliquots taken for liquid scintillation counting. After the tubes had

been allowed to drain for 10 min, the cell pellets were then dissolved overnight in 1 ml of 1 N NaOH at 37°, and finally 0·2-ml aliquots were taken for liquid scintillation counting.

Further 1-ml suspensions of cells in Fischer's medium (10^7 cells) were centrifuged in weighed tubes at 600 *g*, 4° for 10 min, the supernatant poured off and the tubes allowed to drain. After the tubes had been reweighed they were heated to constant weight at 60°.

Metabolism of thymidine. Ascites cells were incubated with [^{3}H]thymidine at the concentrations defined above. After centrifugation the cell pellet was resuspended in 1 ml of ice-cold 80% ethanol and centrifuged at 600 *g* for 10 min. The cell extract was evaporated to dryness and the residue dissolved in 0·1 ml of water. Two-microlitre aliquots were applied to Whatman No. 1 paper together with 2-μl aliquots of cold marker derivatives (10 mM). The derivatives were separated by electrophoresis in 0·05 M citrate–acetate buffer pH 3·75 at 3·5 kV and 80 mA for 30 min. The paper was dried and areas corresponding to the thymidine derivatives, as visualised under u.v. light, were cut out and placed in scintillation vials.

The spots were eluted with 0·1 ml of 0·1 N HCl for 30 min, prior to the addition of 10 ml scintillation fluid and counting.

Pre-labelling of DNA. Tumour-bearing animals (approx wt: 150 g) were given 100 μCi [^{3}H]thymidine (25 Ci/m-mole) i.p. 24 hr prior to administration of drug.

RESULTS AND DISCUSSION

The effects of chlorambucil on the incorporation of [^{3}H]thymidine into the cold acid-soluble pool and into the DNA of Yoshida ascites sarcoma cells, sensitive to the effects of the drug, are shown in Fig. 1. There was a progressive fall of incorporation of [^{3}H]thymidine into the DNA of cells incubated in medium containing 4×10^{-8} M thymidine (Fig. 1c); this inhibition was reversed when cells were incubated in medium containing $2{\cdot}5 \times 10^{-3}$ M thymidine (Fig. 1d). When cells were incubated in the presence of 4×10^{-8} M thymidine the amount of isotope appearing in the cold acid-soluble compartment was unaltered by the drug (Fig. 1a). This indicates that there is no impairment of transport of thymidine across the cell membrane. According to Plageman and Erbe [13] active transport would be the predominant mode of thymidine uptake at a concentration of 4×10^{-8} M, and is the rate-limiting step in the incorporation of thymidine into DNA. Other workers have demonstrated that alkylating agents can affect the uptake of DNA precursors from the incubation medium without affecting DNA synthesis. For example, when Ehrlich ascites cells were exposed to uracil mustard, thymidine uptake stopped within 2 hr, but by 48 hr the DNA content had approximately doubled [14]. The alkylating agent Trenimon also rapidly affects thymidine uptake by Ehrlich ascites cells, within 4 hr of treatment of tumour-bearing animals, but DNA synthesis proceeds unimpaired for at least 10 hr [15, 16]. The possibility exists that the cold acid-soluble pool of the Yoshida cells after 30 min of incubation had reached an equilibrium and that the rate of uptake was slower in treated than

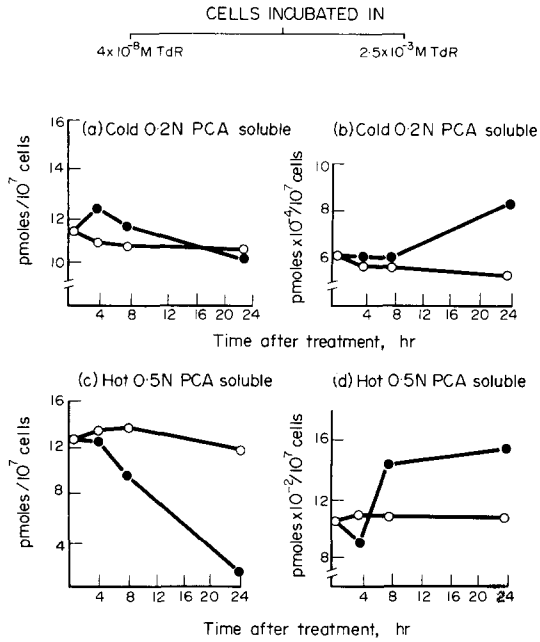

Fig. 1. The distribution of isotope between cold PCA-soluble and hot PCA-soluble extracts of sensitive Yoshida ascites cells, harvested from tumour-bearing animals at the times indicated after administration of chlorambucil (8 mg/kg). Cells (10^7/ml) were incubated in either 4×10^{-8} M [^{3}H]thymidine (a and b) or in 2·5 mM [^{3}H]thymidine (c and d). O——O Untreated cells; ●——● chlorambucil-treated cells (8 mg/kg; each point represents the mean of three experiments.

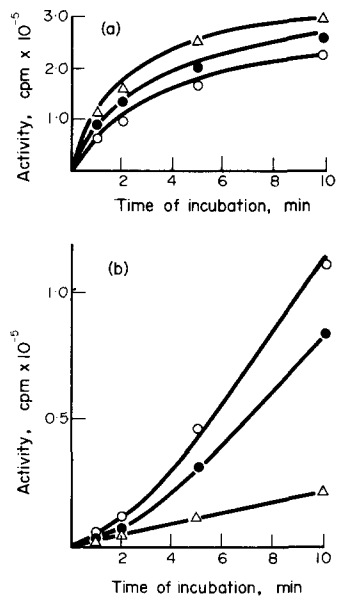

Fig. 2. The effect of alkylating agent treatment on the rate of labelling of (a) acid-soluble and (b) acid-insoluble material during incubation *in vitro* of Yoshida ascites sarcoma cell in Fischer's medium containing 4×10^{-8} M [^{3}H]thymidine. O——O Untreated cells; ●——● chlorambucil-treated cells (8 mg/kg), 8 hr after treatment; △——△ chlorambucil-treated cells (8 mg/kg), 24 hr after treatment.

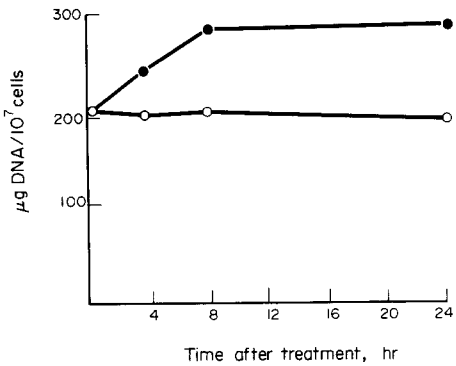

Fig. 3. DNA content of sensitive Yoshida ascites cells following treatment of tumour-bearing animals with alkylating agent. O——O Untreated cells; ●——● chlorambucil-treated cells (8 mg/kg); each point represents the mean of three separate determinations.

untreated cells which would also affect the appearance of label in the DNA. Figure 2 shows the results of experiments to determine the rate of labelling of the acid-soluble and insoluble pools. At times up to 10 min of incubation, it can be seen that the rate of labelling of the acid-soluble pool is not affected by drug treatment but the rate of incorporation into acid-insoluble material is slower in treated cells, confirming the results obtained after 30 min of incubation shown in Fig. 1c. In the first 8 hr following treatment it is not possible to explain the inhibition of thymidine incorporated in terms of an inhibition of DNA synthesis as the amount of DNA per cell is increasing (Fig. 3). An isotope dilution experiment where animals were pre-labelled with isotope, treated, and then cells harvested 4, 8 and 12 hr after treatment also indicated a comparable rate of isotope dilution in treated and untreated Yoshida ascites cells (Table 1).

The increase after 8 hr in the amount of label per cell appearing in the cold PCA soluble compartment (Fig. 1b) is a consequence of the alkylating agent-induced increase in cell volume (Table 2) [11, 17]. There is also an increased incorporation on a per cell basis into the DNA. However, following treatment there is a rise in DNA content per cell (Fig. 3), so that the specific activity of the DNA remains constant.

At extracellular thymidine concentrations of $2{\cdot}5 \times 10^{-3}$ M, transport is predominantly by simple diffusion [13]. Drug treatment does not inhibit either active transport or passive uptake of thymidine. The inhibitory effects of chlorambucil on the incorporation of low concentrations of exogenous thymidine into DNA must, therefore, be located at some other step involved in DNA biosynthesis.

Table 1. Effect of chlorambucil on the decrease of the specific radioactivity of pre-labelled DNA from Yoshida ascites tumour cells

Time after treatment (hr)	Specific activity of DNA [% of value at time zero of treatment (time 0 = 100)]	
	Control	Treated
4	82·4	86·5
8	62·4	68·3
12	52·2	60·1

Table 2. Volume of intracellular water in sensitive Yoshida ascites cells harvested at various times after treatment of tumour-bearing animals with chlorambucil (8 mg/kg)

Time after treatment (hr)	Intracellular water (μl/10^7 cells)	
	Control	Treated
0	12·0	
3	12·1	12·5
6	11·8	11·4
9	12·1	21·7
24	11·6	22·3

Each value represents the mean of 6 determinations. Overall scatter ± 10 per cent.

An examination of the distribution of [^{3}H]thymidine and its phosphorylated derivatives was made. It has been reported that alkylating agents do not affect the phosphorylation of thymidine to TTP [18, 19]. Table 3 shows the distribution of label after incubation in medium containing 4×10^{-8} M [^{3}H]thymidine: over 50 per cent of the total label was present as TTP. At no time after treatment was there any inhibition of the conversion of thymidine to TTP. After incubation in the presence of $2{\cdot}5 \times 10^{-3}$ M [^{3}H]thymidine, over 80 per cent of the total label was present as thymidine (Table 4); again drug treatment was without effect upon the distribution of label. It is not possible, therefore, to explain the difference in incorporation of [^{3}H]thymidine into DNA when cells are incubated in low or high extracellular concentrations of isotope in terms of drug effects on transport or on conversion of thymidine to its phosphorylated derivatives. The possibility that in low thymidine concentration the endogenous pool is expanding following drug treatment and hence diluting the label can also be excluded. It has been found that the TTP pool in untreated Yoshida cells is 44 nmoles/10^9 cells and that after 8 hr this has increased only to 49 nmoles/10^9 cells [20].

The remaining possibility to explain an inhibition of [^{3}H]thymidine incorporation whilst DNA synthesis continues is that chlorambucil precludes the

Table 3. Distribution of ^{3}H-label between thymidine nucleotides after incubation of Yoshida ascites sarcoma cells (10^7 cells/ml) in 4×10^{-8} M [^{3}H]thymidine. Cells were harvested from tumour-bearing animals at the times indicated after administration of chlorambucil (8 mg/kg)

Time after treatment (hr)	% Of total label appearing as			
	TdR	TMP	TDP	TTP
Control	4	25	16	55
3	3	22	14	61
9	10	4	10	76
24	11	9	12	68

Total concentration of isotope in untreated cells = approx 10 pmoles/10^7 cells (see Fig. 2).

Each value represents the mean of four determinations. Overall scatter ± 10 per cent.

Table 4. Distribution of ^{3}H-label between thymidine nucleotides after incubation of Yoshida ascites sarcoma cells (10^7 cells/ml) in $2{\cdot}5 \times 10^{-3}$ M [^{3}H]thymidine. Cells were harvested from tumour-bearing animals at the times indicated after administration of chlorambucil (8 mg/kg)

Time after treatment (hr)	% Of total label appearing as			
	TdR	TMP	TDP	TTP
Control	81·0	15·0	1·6	2·4
3	87·0	9·4	1·5	2·1
9	85·0	8·7	2·3	4·0
24	82·0	14·5	1·5	2·0

Total concentration of isotope in untreated cells is approx 6×10^4 pmoles/10^7 cells (see Fig. 2).

Each value represents the mean of four determinations. Overall scatter ± 10 per cent.

utilisation of TTP, derived from exogenous thymidine, for DNA synthesis. It may be that TTP synthesized by the *de novo* route is compartmentalized (physically or metabolically) from TTP derived from the "salvage" pathway, and that chlorambucil interferes with the equilibration of these separate pools: this is reversed by high extracellular concentrations of thymidine. There is some evidence from recent work with eucaryotic cells that nucleoside triphosphates are compartmentalized in intracellular pools [21]. Further, studies with bacterial cells point to the existence of a semiconservative DNA synthesising system either functionally or physically compartmentalised from a DNA repair system [22–24].

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