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COMPARATIVE EFFECTS OF ALKYLATING AGENTS AND OTHER ANTI-TUMOUR AGENTS ON THE INTRACELLULAR LEVEL OF ADENOSINE 3′,5′-MONOPHOSPHATE IN WALKER CARCINOMA

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Abstract—The ability of a number of anti-tumour agents to elevate the intracellular level of adenosine 3′,5′-monophosphate (cyclic AMP) in Walker carcinoma has been investigated. Of these agents HN2, chlorambucil, merophan, CB 1954, and *cis*-dichloro diammine Pt II [*cis*-$Cl_2(NH_3)_2$ Pt II) caused appreciable elevations of the cyclic nucleotide. These compounds are all regarded as having a typical alkylating agent spectrum of action. A linear relationship was obtained between the intracellular cyclic AMP level produced by a given dose of *cis*-$Cl_2(NH_3)_2$ Pt II and the observed percentage inhibition of cell growth. There was a rapid elevation of cyclic AMP in response to chlorambucil which reached its peak within 1 hr of treatment. This preceded the inhibition of thymidine incorporation which reached 50 per cent by 7 hr. 1,3-*Bis*-(2-chloroethyl)1-nitrosourea (BCNU) and (±) 1,2-*bis*-(3.5-dioxopiperazin-1-yl)propane (ICRF 159) had no effect on the intracellular level of cyclic AMP in Walker carcinoma at any dose level studied. The cyclic 3′,5′-nucleotide phosphodiesterase from bone marrow and intestinal mucosa, two tissues susceptible to alkylating agents, has been investigated. This enzyme behaves kinetically as if two separate activities exist, one with a low affinity for the substrate and the other with a high affinity. The distribution of the two forms of the enzyme from these tissues agrees with the correlation previously obtained between sensitivity to alkylating agents and a high percentage of the low K_m form of the enzyme.

Neoplastic transformation is often accompanied by aberrations in the adenosine 3′,5′-monophosphate (cyclic AMP) system in both virally [1–3] and chemically [4] transformed cells. Thus, a number of transformed mammalian cells in culture have lower basal levels of cyclic AMP than the corresponding normal cells [5, 6]. Addition to transformed cells, of cyclic AMP, or agents which elevate the intracellular level of cyclic AMP, causes growth inhibition and cell kill both *in vitro* [7–9] and *in vivo* [10–12] and in some cases, morphological reversion and differentiation [13–15].

It has been shown previously that the anti-tumour alkylating agents cause an elevation of intracellular cyclic AMP in Walker carcinoma cells at doses which produce cell kill [16]. This seems to be due to their ability to inhibit selectively the high affinity form of the cyclic 3′,5′-nucleotide phosphodiesterase (adenosine 3′,5′-monophosphate phosphohydrolase, EC 3.1.4.c). The inactive monofunctional analogue of an active difunctional agent was shown to have no effect on cyclic AMP levels at toxic doses. It was suggested from these data that the effects of the alkylating agents might be mediated, at least in part, by the elevation of cyclic AMP levels.

While a destructive action on chromosomes is thought to play a part in tumour growth inhibition by alkylating agents, Koller [17] has distinguished two effects, one structural in which alkylating agents are believed to cause chromosome abberation by direct action, and one functional in which mitosis is blocked, often temporarily. This latter effect might be mediated by cyclic AMP. Both alkylating agents and exogenous cyclic AMP cause an inhibition of mitosis by causing a decrease in the rate of DNA synthesis [18, 19] which leads to a prolongation of the *S* phase of the cell cycle [19–21].

The inhibitory effects of alkylating agents on antibody production [22] could also be mediated via cyclic AMP since it is known to lead to a suppression of the immune response [23, 24].

To investigate the generality of the phenomenon of elevated cyclic AMP levels in cells treated with alkylating agents, the effects of a range of anti-tumour agents on the cyclic AMP system have been studied. Also the kinetics of the cyclic nucleotide phosphodiesterase from bone marrow and intestinal mucosa have been determined to see if the previously observed correlation [16] between a high percentage of the low K_m form of the enzyme and sensitivity to alkylating agents also applies in these sensitive tissues.

MATERIALS AND METHODS

Chemicals. 5-Methyl-[^{3}H]thymidine (sp. act. 5 Ci/m-mole) and [8-^{3}H]cyclic AMP (sp. act. 27·5 Ci/m-mole) were purchased from the Radiochemical Centre, Amersham. Unlabelled cyclic AMP was obtained from BDH Chemicals, Poole, Dorset. Scintillation fluid NE 233 was purchased from Nuclear Enterprises Ltd., Edinburgh and PCS solubilizer from Hopkin• & Williams, Romford. All the alkylating

agents used in this study were synthesized in the Chester Beatty Research Institute. The 4-ethoxy metabolite of cyclophosphamide was kindly provided by Dr. P. J. Cox, Chester Beatty Research Institute. ICRF 159 was a generous gift of Drs. A. Creighton and K. Hellmann, Imperial Cancer Research Fund. BCNU was kindly supplied by Dr. H. Woods, National Cancer Institute, Washington. All other chemicals were available commercially.

Cell culture. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum, under an atmosphere of 10% CO_2 in air as described previously [25]. A line highly sensitive to alkylating agents, Walker S (WS), was derived from the Walker ascites carcinoma. A highly resistant line, WM III, was derived *in vitro* from a resistant line, WM I [16], by repeated treatment with progressively increasing doses of chlorambucil. The resistant cells were treated with a dose of 100 μg/ml of chlorambucil every 10 days, to maintain resistance.

Drug treatment. Cells were taken from rapidly-growing cultures and resuspended in fresh medium at 5×10^5 cells per ml. Drug solutions were made up at 100 times the required concentration and 1 ml of drug solution was added to aliquots of 100 ml of cell suspension. The alkylating agents and BCNU and ICRF 159 were dissolved in dimethyl sulphoxide and *cis*-$Cl_2(NH_3)_2$ Pt II in dimethyl acetamide. Controls received solvent alone. Cells treated with ICRF 159 were incubated continuously in the presence of the drug. Treatment with other agents was limited to 1 hr, after which time the cells were centrifuged at 300 *g* for 3 min, washed and resuspended in fresh medium. The cell suspensions were then incubated at 37° for the periods stated in the tables.

The effect of drug treatment on cell growth was estimated as previously described [25]. Drugs were added to cells suspended at 10^5/ml in culture medium, and the cells were then either incubated for 1 hr, washed and resuspended at the same density (alkylating agents) or plated out immediately in the presence of ICRF 159. In either case the suspensions were dispersed in 200 μl amounts into the wells of a 96-well plastic plate and cell counts were made every 24 hr on several wells of each series. Counts were used to construct growth curves from which percentage inhibition of growth was estimated [25].

Adenosine 3',5'-monophosphate phosphodiesterase preparation. Both bone marrow and intestinal mucosal cells were obtained from Wistar Chester Beatty male rats. Animals were killed by cervical dislocation and the small intestines were dissected out into saline. The gut was then cut longitudinally and rinsed in saline. The mucosa was removed by scraping with a scalpel and the cells were washed several times in saline.

The femurs were then dissected out of the same animals and the epiphysis was clipped off either end of each bone. The bone marrow was then blown out, using a syringe connected to the bone by a rubber tube. The bone marrow was washed several times in saline.

Both types of cells were then suspended in cold 0·25 M sucrose and treated with a 20-Kc MSE sonic oscillator (1 sec/ml). The lysate was centrifuged at 100,000 *g* for 1 hr and the supernatant fluid was used for the assay of phosphodiesterase after dialysis at 4° against 100 mM Tris–HCl, pH 8·1, containing 10 mM $MgSO_4$.

Assay of adenosine 3',5'-monophosphate phosphodiesterase. The determination of adenosine 3',5'-monophosphate phosphodiesterase has been previously described [16]. Essentially [8-^{3}H]cyclic AMP was separated from the product of hydrolysis, [8-^{3}H]5'-AMP by means of thin layer chromatography (t.l.c.) and the region of the t.l.c. plate corresponding to 5'-AMP was removed and the radioactivity estimated by liquid scintillation spectrometry in NE 233 scintillation fluid.

Protein was estimated by the method of Lowry *et al.* [26] using bovine serum albumin as the standard.

The incubation period was chosen to stop the reaction at less than 10% cyclic AMP hydrolysed. Under these conditions the rate of cyclic AMP hydrolysis was proportional to protein concentration and to elapsed time. Data are expressed as nmoles of [^{3}H]cyclic AMP hydrolysed per min/mg of protein.

Cyclic AMP assay. The estimation of cyclic AMP was as previously described [16]. The concentration of cyclic AMP was determined by means of an assay kit (Radiochemical Centre, Amersham). A standard curve was performed for each determination. Radioactivity was measured in PCS solubilizer.

Effect of chlorambucil on the uptake of thymidine into Walker carcinoma. Walker cells ($2·5 \times 10^5$ ml) were incubated with shaking at 37° with either chlorambucil (5 μg/ml) or solvent as control. At various times, aliquots of the cells were removed and a portion of each was used for the cyclic AMP determination. The remainder was incubated for 1 hr at 37° with tritiated thymidine (5 μCi/ml). At timed intervals 1 ml samples were filtered through a fibre glass disc (Whatman GF/C, 2·5 cm). The cells on the disc were washed with 10 vol 0·9% NaCl solution. The discs were dried at 70° for 1 hr and scintillation fluid (naphthalene, PPO and diMePOPOP, in ethanol, dioxan and toluene) was added directly to them for the assay of radioactivity by liquid scintillation counting (Packard Tricarb, Model 3375).

RESULTS

The results given in Table 1 show the effect of several alkylating agents on the cyclic AMP levels and the viability of sensitive Walker carcinoma cells in culture. The values for chlorambucil previously reported [16] are also included for comparison. For both the aromatic nitrogen mustards, chlorambucil and merophan and also for the aliphatic congener HN2, the percentage increases in intracellular cyclic AMP for a given degree of growth inhibition are remarkably similar. Thus a 100 per cent inhibition of cell growth corresponds approximately to a doubling in cyclic AMP levels.

The relationship between the time required to elevate cyclic AMP levels in Walker cells in the presence of 5 μg/ml of chlorambucil, and the inhibition of thymidine uptake into DNA, is shown in Fig. 1. Thus, while cyclic AMP levels are raised rapidly, reaching a maximum between 45 and 60 min after treatment, the effect on thymidine incorporation is much slower, only 50 per cent inhibition being reached by 7 hr.

The results presented in Table 1 show that the ethoxy derivative of 4-hydroxycyclophosphamide, which

Table 1. Effect of alkylating agents on the intracellular level of cyclic AMP and viability of Walker carcinoma cells

Compound	Dose (μg/ml)	Increase in cyclic AMP level (%)	Growth inhibition (%)
Merophan*	0·5	118	99
	0·1	76	83
	0·05	18	63
HN2†	0·1	108	100
	0·05	68	97
Chlorambucil*	5·0	114	99
	1·0	72	80
	0·5	21	50
4-Ethoxycyclophosphamide†	10·0	37	100
CB 1954*	1·0	96	100
	0·4	67	100
	0·05	22	97

* Cyclic AMP values 8 hr after treatment.
† Cyclic AMP values 24 hr after treatment.

is thought to give rise to the active metabolite of cyclophosphamide [27], is not very efficient at raising cyclic AMP levels in sensitive Walker cells when measured 24 hr after treatment. This is surprising since the ring size and substituents of this compound bear a close resemblance to that of cyclic AMP itself, and as such may be thought to effectively interact with the active site of the cyclic nucleotide phosphodiesterase. However, this ethoxy analogue may require hydrolysis back to the 4-hydroxy compound before it can fulfil this prediction and this hydrolysis would not be expected to be very rapid. It is possible that the increase in cAMP observed at a dose of 4-ethoxy cyclophosphamide giving 100 per cent growth inhibition is small, compared with the rise caused by an equitoxic dose of chlorambucil, because of differences in the time-course of the changes after treatment. This may also apply to the other compounds mentioned below, where single determinations of cAMP have been made.

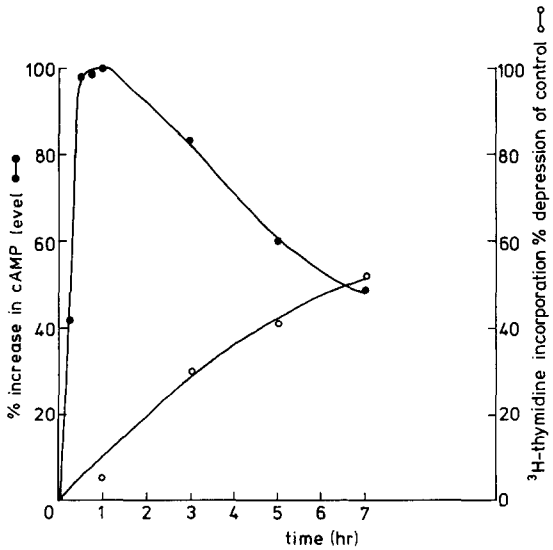

Fig. 1. Effect of continuous incubation with 5 μg/ml of chlorambucil on the intracellular cyclic AMP and the incorporation of labelled thymidine into the DNA of Walker tumour cells.

5-Aziridinyl-2,4-dinitrobenzamide (CB 1954) is a potent and selective inhibitor of the growth of the Walker carcinoma [28]. The results given in Table 1 demonstrate an elevation of the intracellular cyclic AMP by CB 1954 in this tumour at concentrations which cause an inhibition of cell growth. This compound therefore differs from other monofunctional alkylating agents, such as the *N*-ethyl analogue of chlorambucil, in being both an effective anti-tumour agent and also in raising cyclic AMP levels.

Cis-dichloro diammine Pt II [*cis*-$Cl_2(NH_3)_2$ Pt II] is very effective in the treatment of some transplantable animal tumours such as the Walker carcinoma [29, 30]. The effect of this compound on the cyclic AMP levels in this tumour is shown in Table 2. There is a linear dose response relationship between intracellular cAMP level and the percentage inhibition of growth similar to that previously reported for chlorambucil [16].

Another anti-tumour agent currently undergoing intensive clinical investigation is (±)1,2-*bis*(3,5-dioxopiperazin-1-yl) propane (ICRF 159). Table 2 shows the intracellular levels of cyclic AMP in Walker carcinoma 8 hr after treatment with a range of concentrations of ICRF 159, and the percentage inhibition of growth obtained. This compound has no effect on the basal level of cyclic AMP in this tumour, even at a concentration which produces 94 per cent inhibition of cell growth. The effect of another anti-tumour agent 1,3-*bis*(2-chloroethyl)-1-nitrosourea (BCNU) on the intracellular level of cyclic AMP in Walker carcinoma 8 hr after treatment with a range of concentrations sufficient to produce up to 100 per cent inhibition of cell growth is also shown in Table 2. Again no elevation of cyclic AMP levels is observed.

The rate of hydrolysis of cyclic AMP by bone marrow cells is shown in Fig. 2. The data are expressed by means of a Hofstee plot [31] in which the slope is the negative value of the apparent K_m value and the intercept on the ordinate is the value of the apparent V_{max}. Incubation conditions with respect to both time and enzyme concentration were carefully chosen

Table 2. Effect of other anti-tumour agents on the intracellular level of cyclic AMP and cell viability of Walker carcinoma

Compound	Dose (μg/ml)	Cyclic AMP (μM)	Growth inhibition (%)
cis $Cl_2(NH_3)_2$ Pt II*	0·5	2·9	96
	0·25	2·65	88
	0·1	2·1	56
	0·05	1·95	40
	0·00	1·95	—
ICRF 159†	100	1·98	94
	50	1·95	92
	25	1·90	79
	10	1·76	46
	0	1·96	—
BCNU†	100	1·24	100
	20	1·26	99
	10	1·25	96
	0	1·22	—

* Levels measured 6·5 hr after treatment.
† Levels measured 8 hr after treatment.

to ensure linearity of cyclic AMP hydrolysis. A compilation of the kinetic data for the cyclic nucleotide phosphodiesterase from bone marrow, intestinal mucosa and a 150-fold resistant subline of the Walker carcinoma is shown in Table 3. In each case the data may be fitted with two lines to a first approximation, which suggests either the presence of two enzyme activities for each tissue, or a negatively cooperative control system [32]. The apparent K_m values for each form of the enzyme in each tissue do not differ significantly. However, the percentage of the total activity contributed by the low K_m form of the phosphodiesterase, while being relatively high in bone marrow and intestinal mucosa (28 and 20 per cent respectively), accounts for only a small proportion (8·5 per cent) of the total enzyme activity of the resistant Walker tumour.

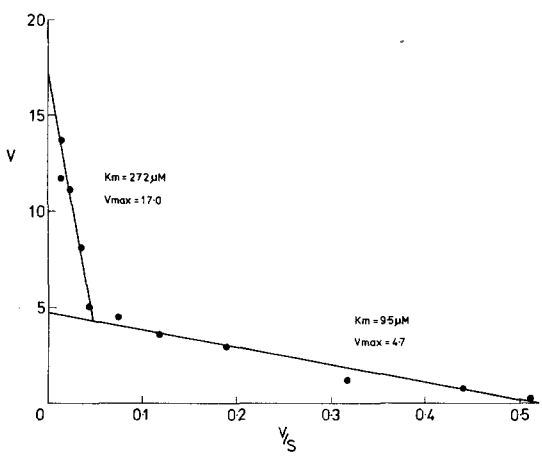

Fig. 2. Hofstee plot [31] for the hydrolysis of cyclic AMP by bone marrow cells. Samples were analysed as in methods and the initial velocities were determined at substrate concentrations ranging from 0·2 to 1000 μM. V = initial velocity (nmoles of cyclic AMP hydrolysed/min/mg protein); S = substrate concn (μM).

DISCUSSION

In a previous communication [16], it was shown that the bifunctional alkylating agent, chlorambucil, caused an increase in the intracellular level of cyclic AMP in sensitive, but not resistant, Walker carcinoma cells in culture. The increase observed was comparable to that produced by the phosphodiesterase inhibitor, theophylline, at equitoxic doses and was shown to be associated with inhibition of a high affinity form of phosphodiesterase.

In the present publication it was shown that 5 μg/ml of chlorambucil caused an immediate rise in intracellular cyclic AMP in Walker cells, which reached a maximum within 1 hr. This effect preceded the effect of the drug on thymidine incorporation into DNA. The selective inhibition of thymidine uptake has been one of the earliest detectable effects of alkylating agents previously reported [34]. The fact that the effect on cyclic AMP preceded inhibition of thymidine incorporation suggests that cyclic AMP may mediate an effect of chlorambucil on DNA synthesis [35].

Several other pieces of evidence support the hypothesis that cyclic AMP might mediate the effects

Table 3. Kinetic parameters for the hydrolysis of cyclic AMP by cyclic nucleotide phosphodiesterase

Cell type	K_{mI} (μM)	V_{maxI} (nmole/min/mg protein)	K_{mII} (μM)	V_{maxII} (nmoles/min/mg protein)	% of V_{maxII} to total activity
Bone marrow	272	16·9	9·5	4·65	28
Intestinal mucosa	369	6·0	6·1	1·20	20
WM III	116	3·3	4·1	0·28	8·5

of alkylating agents. For example, HeLa cells show maximum sensitivity to mustard gas when at the *G1/S* interphase [21], while human lymphoid cells undergo a mitotic delay following treatment during *S* phase, with the cyclic nucleotide phosphodiesterase inhibitor 1-methyl-3-isobutyl xanthine [33]. In addition, cyclic AMP itself is known to inhibit thymidine incorporation into DNA [19, 36]. To investigate the generality of the observed effect on cyclic AMP, the levels of the nucleotide were measured in Walker carcinoma cells 8 hr after treatment with a variety of other antitumour agents. While a number of substances commonly regarded as typical alkylating agents caused appreciable increases in intracellular cyclic AMP, no such increase was produced by some other antitumour agents.

In their spectrum of anti-tumour action, the active platinum compounds closely resemble the alkylating agents [37]. The similarity between these two classes of compounds is also reflected by similar effects on the cyclic AMP system. Thus, a linear relationship is obtained between the intracellular level of cyclic AMP and the percentage inhibition of cell growth produced by *cis*-$Cl_2(NH_3)_2$ Pt II in Walker cells. This suggests either a direct or indirect relationship between these two effects.

The chloroethyl-substituted nitrosoureas are effective against a broad range of animal tumours. Although they can act as alkylating agents, since they decompose readily under physiological conditions to generate a carbonium ion [38], they appear to be quite different in many of their biological features [34]. With regard to its effect on intracellular cyclic AMP levels, BCNU would appear to differ from a typical alkylating agent, since no detectable rise in level occurred 8 hr after treatment, even with a dose which caused 100 per cent inhibition of cell growth. This contrasts with those drugs acting by an alkylating type of mechanism which were found to cause a doubling of cyclic AMP levels at doses which caused a comparable degree of growth inhibition.

Studies with ICRF 159 show it to be an antimitotic agent which induces the formation of mono- and binuclear giant cells in culture, similar in many respects to those produced by the nitrogen mustards [39]. This agent, however, also has no apparent effect on the intracellular level of cyclic AMP in Walker cells in culture at any of the concentrations which produce an inhibition of cell growth.

The alkylating agents are characterized by a high order of selective action against certain proliferative tissues: haematopoietic cells in bone marrow and lymphoid organs, the intestinal mucosa, and some neoplastic tissues [40]. It has been shown previously [16] that in those tumours which are naturally resistant to the growth inhibitory properties of the alkylating agents, the apparent V_{max} of the low K_m form of the cyclic nucleotide phosphodiesterase is less than 10 per cent of the total activity. Further, the percentage of this form decreases from 38 per cent of the total in Walker cells sensitive to alkylating agents to 8·5 per cent in a 150-fold resistant line. This correlation was maintained with the cyclic nucleotide phosphodiesterase from bone marrow and intestinal mucosa in which the contribution of the low K_m form to the total activity was 28 and 20 per cent respectively. Recent results suggest that a specific form of this enzyme is lost in resistant tumours.*

Thus an increase in cyclic AMP appears to be specifically associated with compounds acting by an alkylating type of mechanism and the evidence presented in this and previous papers suggests that this effect may be important in their mechanism of cell killing. Experiments are now in progress to determine further the relevance of the effect on cyclic AMP to the biochemical mechanisms involved in growth inhibition by alkylation.

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