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THE EFFECT OF ALKYLATING AGENTS ON THE ACTIVITY OF ADENOSINE 3′,5′-MONOPHOSPHATE-DEPENDENT PROTEIN KINASE IN WALKER CARCINOMA CELLS

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Abstract—The effect of some alkylating agents on the activity of the adenosine 3′,5′-monophosphate (cyclic AMP)-dependent protein kinase has been studied in Walker cells sensitive and resistant to the cytotoxic action of such agents. Chlorambucil (5 μg/ml) caused an activation of the cAMP-dependent protein kinase in sensitive Walker carcinoma cells which reached a maximum 1.5 hr after drug addition. Sephadex gel chromatography indicated that during this activation, the catalytic subunit of the protein kinase was released from the holoenzyme to the same extent as that measured in the crude supernatant of the tumour cells. The degree of activation was equivalent to that produced by 100 μg/ml of N^6,$O^{2'}$-dibutyryl cAMP. In contrast, the monofunctional *N*-ethyl analogue of chlorambucil had no effect on the cAMP-dependent protein kinase at a dose of 250 μg/ml. The protein kinase activity ratio in sensitive cells increased with increasing doses of chlorambucil and reached a maximal activation at a concentration of 5 μg/ml, which was sufficient to cause complete inhibition of tumour cell growth. A much larger dose of chlorambucil (100 μg/ml) was required to cause activation of the kinase in Walker cells resistant to this agent. Chlorambucil (25 μg/ml) also caused an activation of the cAMP-dependent protein kinase in TLX5 cells, though the time scale of the activation differed for that found in Walker cells. Both merophan and 5-aziridinyl-2,4-dinitrobenzamide (CB 1954) caused an increase in the protein kinase activity ratio of sensitive Walker cells. The increase caused by CB 1954 could be abolished by 4-amino-2-phenylimidazole-5-carboxamide (2-phenyl-AIC), which reverses the tumour growth inhibitory action of CB 1954. The degree of stimulation of the cytosolic protein kinase by saturating concentrations of cAMP, and the apparent dissociation constant for cAMP bound to protein kinase decreased with increasing resistance of the cell lines to alkylating agents. These results suggest that the biological effect of the increase in cAMP in sensitive Walker cells induced by the alkylating agents is mediated through a protein kinase.

Investigations into the mode of action of the anti-tumour alkylating agents have shown that bifunctional derivatives, which are therapeutically effective, cause an elevation of the intracellular level of adenosine 3′,5′-monophosphate (cyclic AMP) in Walker carcinoma cells in tissue culture [1, 2]. Of the monofunctional agents tested only 5-aziridinyl-2,4-dinitrobenzamide (CB 1954) produced a comparable rise in cAMP levels [2]. The possible therapeutic importance of the rise in cAMP has been investigated by comparing factors involved in cAMP metabolism in Walker cells sensitive and resistant to alkylating agents. The cAMP phosphodiesterase, which hydrolyzes cAMP to 5′AMP, shows marked alteration in cells resistant to either chlorambucil or melphalan. This enzyme displays kinetics indicative of a high and a low affinity form. In resistant cells there is a reduction in the activity of the high affinity form of the enzyme with no corresponding reduction of the low affinity form [3]. When the multiple forms of the enzyme were separated by Sepharose 6B gel chromatography a specific loss of high mol. wt forms was evident in some resistant cells [4, 5]. The quaternary structure of the enzyme would also appear to be altered in resistant cells, since there is a shift in pH optima and also a 10-fold difference in the K_i values for the competitive inhibitor theophylline for the enzyme from sensitive and resistant cells [5].

Cyclic AMP is believed to exert its biological effect by the activation of a cAMP-dependent protein kinase (ATP: protein phosphotransferase; EC 2.7.1.37) [6, 7]. The initial step in such an activation involves the binding of cAMP to the regulatory subunit (R) of the holenzyme (R–C) followed by dissociation of the latter. The extent of binding of cAMP to specific cytosolic sites has been shown to be reduced in Walker cells made resistant either to CB 1954 or chlorambucil [8]. The biological importance of this loss of binding protein was shown by the cross-resistance of these cell lines to N^6, $O^{2'}$-dibutyryl cAMP (dibutyryl cAMP) [8].

These results suggest that the action of cAMP may be mediated by a protein kinase. Indeed the available evidence indicates that all the effects of cAMP are mediated through its ability to control the level of the free catalytic subunit (C) [6, 9]. The present paper reports results on the effect of pharmacological doses of alkylating agents, on the activity of cAMP-dependent protein kinase in Walker cells in tissue culture.

MATERIALS AND METHODS

[γ-^{32}P]ATP (sp.act. 2.25 Ci/m-mole) and [8-^{3}H]cAMP were purchased from the Radiochemical Centre; Amersham. Histone (type II-A), unlabelled cAMP and dibutyryl cAMP were obtained from Sigma Chemical Company; London. Scintillation fluid NE 233 was purchased from Nuclear Enterprises Ltd; Edinburgh. Cellulose ester filters were from Millipore Corporation; London and ATP from Boehringer Corporation; London. 1-Methyl-3-isobutylxanthine was purchased from Aldrich Chemical Company; London. All the alkylating agents used were synthesized at the Chester Beatty Research Institute.

Cell culture. Cell lines were maintained in static suspension culture in Dulbecco's modified Eagle's medium, supplemented with 10% foetal calf serum, under an atmosphere of 10% CO_2 in air. The conditions for the establishment and maintenance of resistant Walker cells has previously been described [8].

Drug treatment. Cells were taken from rapidly growing cultures and resuspended in fresh medium at 5×10^5 cells/ml. Drug solutions were made up in dimethyl sulphoxide at one hundred times the required final concentration and were added to the cell suspensions incubated at 37° to give final concentrations of dimethyl sulphoxide of 1%. The sensitivity of the various cell lines to drug treatment has previously been reported [8].

Cyclic AMP binding assay. The binding of cAMP to proteins was measured by a modification of the method of Gilman [10]. At appropriate times after drug treatment, cells were removed by centrifugation at 300 g for 3 min, followed by washing in 0.9% NaCl, and recentrifugation. All subsequent operations were carried out at 0–4°. The cell pellets were sonicated with a 20-kHz MSE sonic oscillator in 10 mM Tris–HCl, pH 7.6, containing 250 mM sucrose and 1 mM $MgCl_2$. The supernatant fraction obtained after centrifugation at 100,000 *g* for 1 hr was used for the determination of cAMP binding activity. The reaction mixture (0.2 ml final volume) contained 50 mM sodium acetate buffer (pH 4.0), 3 mM theophylline, and 80 nM [8-^{3}H]cAMP. The reaction was initiated by addition of 100 μl of tumour cytosol, and binding was allowed to take place for at least 2 hr at 4°. Protein-bound cAMP was determined by filtration through a 25-mm cellulose ester filter (0.45 μm pore size) and the filter containing protein-bound [8-^{3}H]cAMP was dissolved in 1 ml of 2-(methoxy)-ethanol and the radioactivity was determined in a toluene, PPO scintillation mixture using a Tracer Lab counter. Protein concentrations were determined by the method of Lowry *et al.* [11] using bovine serum albumin as a standard.

Protein kinase activity ratio. The method of Corbin and Reimann [12] was used to determine the protein kinase activity ratio. Washed cells, which were obtained as above, were suspended in 10 mM potassium phosphate buffer, pH 6.5, containing 10 mM EDTA, 0.5 mM 1-methyl-3-isobutylxanthine and 500 mM NaCl (or 50 mM NaCl) and were disrupted by freezing in an acetone–cardice bath, followed by thawing and homogenization with a Teflon–glass homogenizer. This procedure was necessary because of the difficulty of rupturing Walker cells by conventional methods. This operation was performed rapidly because of the danger of 1-methyl-3-isobutylxanthine causing elevation of cAMP levels [13]. The homogenate was centrifuged at 12,000 *g* for 20 min at 4° and the protein kinase activity of the supernatant was assayed in the presence and absence of 2 μM cAMP. The reaction mixture (80 μl final vol) contained final concentrations of 0.21 mM [γ-^{32}P]ATP (sp. act. 100 cpm/pmole), 25 mM NaF, 0.5 mg histone (type II-A), 0.125 mM 1-methyl-3-isobutylxanthine, and 3.75 mM magnesium acetate in 50 mM potassium phosphate buffer, pH 6.8. The mixture was incubated at 30° and the reaction was initiated by the addition of 20 μl of the supernatant fluid. After incubation for a suitable time (routinely 5 min after the linearity of the reaction had been established) the reaction was terminated by pipetting 50 μl of the mixture onto Whatman 3MM filter paper discs (2.3 cm dia). The discs were dropped immediately into ice-cold 10% trichloracetic acid (5 ml/filter disc), washed with trichoroacetic acid (four times), ethanol and ether. The radioactivity on the disc was determined in NE233 scintillation fluid using a Tracer Lab counter. Assays were performed in triplicate. The activity of the protein kinase was expressed as the ratio of [^{32}P]incorporated into histones in the absence (−cAMP) and presence (+cAMP) of cAMP.

Sephadex chromatography. To separate the protein kinase subunits, the supernatant fraction derived from the tumour cell homogenate was chromatographed on a Sephadex G-100 column (24 × 1 cm) [14]. The void volume was monitored using blue dextran. The column was equilibrated with 10 mM potassium phosphate buffer, pH 6.5, containing 10 mM EDTA and 500 mM NaCl, and 0.5 ml of the supernatant fraction was applied to the column. The flow rate was 8 ml/hr. Seventy fractions (0.5 ml each) were collected and the enzyme activity (+ or − cAMP) was determined in 20 μl of each fraction.

RESULTS

According to the model of protein kinase activation [6, 7], elevation of the intracellular level of cAMP should result in occupation of sites on the regulatory subunit of a protein kinase in proportion to the degree of kinase activation. Thus subsequent incubation of this binding protein with a saturating concentration of [8-^{3}H]cAMP should result in less binding than that seen in extracts from untreated cells. This can be seen in the results shown in Fig. 1a which shows that specific cAMP binding by cytosol of the sensitive Walker tumour decreases 1 hr after chlorambucil treatment to 50 per cent of the binding activity of the control. This corresponds to the time at which the cAMP level of the cells has reached a maximum [2]. The protein kinase activity ratio in sensitive cells at various times after treatment with either 5 μg/ml of chlorambucil or 250 μl/ml of the therapeutically inactive monofunctional *N*-ethyl analogue is shown in Fig. 1b. The protein kinase activity ratio (−cAMP/+cAMP) is the ratio between the protein kinase activity of the supernatant fraction assayed in the absence of added cAMP and that measured in the presence of saturating concentrations of cAMP. Under the standard assay conditions the

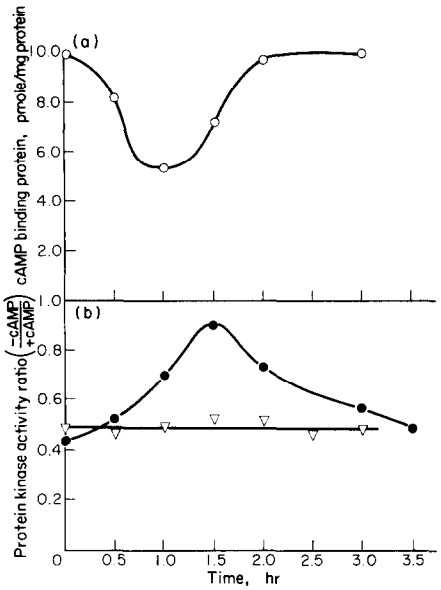

Fig. 1. (a) cAMP binding activity of sensitive Walker cell cytosolic protein after treatment with chlorambucil (5 μg/ml). (b) Changes in the protein kinase activity ratio in Walker cells after treatment with either 5 μg/ml of chlorambucil (●——●) or 250 μg/ml of the *N*-ethyl analogue (▽——▽).

incorporation of [^{32}P]phosphate from [γ-^{32}P]ATP into total histone increases linearly with reaction time up to 15 min. The results in Fig. 1b show that while the protein kinase activity ratio is unaffected by the monofunctional analogue of chlorambucil, there is a time-dependent increase in the ratio in the presence of chlorambucil, which reaches a maximum 204 per cent of control, 1.5 hr after chlorambucil addition.

In order to substantiate the reliability of the protein kinase activity ratio as an index of cAMP-dependent protein kinase activity, Sephadex G-100 chromatography was employed to separate the subunits [14]. The elution profile of the enzyme 1.5 hr after administration of 5 μg/ml of chlorambucil or 100 μg/ml of dibutyryl cAMP, or solvent alone, is shown in Fig. 2. In the control sample shown in Fig. 2a about 50 per cent of the cAMP-dependent protein kinase is in the R–C form (fractions 40–46) and 50 per cent in the C form (fractions 48–60). The relative amounts of R–C and C in the control directly correlate with the −cAMP/+cAMP ratio of 0.45 (Fig. 1b). Within 1.5 hr of chlorambucil administration there is a change in the profile (Fig. 2b) with less cAMP-dependent protein kinase in the R–C form and a corresponding increase in the C form. This change is due to an elevation in endogenous cAMP which binds to the R unit of the R–C form of the kinase and results in release of C [9]. This profile correlates well with the −cAMP/+cAMP ratio of 0.9 (Fig. 1). A similar profile to that in Fig. 2b is shown in Fig. 2c for cells treated with 100 μg/ml of dibutyryl cAMP. In this case the protein kinase activity ratio is about 0.8. This indicates that 1.5 hr after administration of either chlorambucil or dibutyryl cAMP, the cAMP-dependent protein kinase is almost fully activated.

The time course for stimulation of the kinase by dibutyryl cAMP is shown in Fig. 3. Maximal stimu-

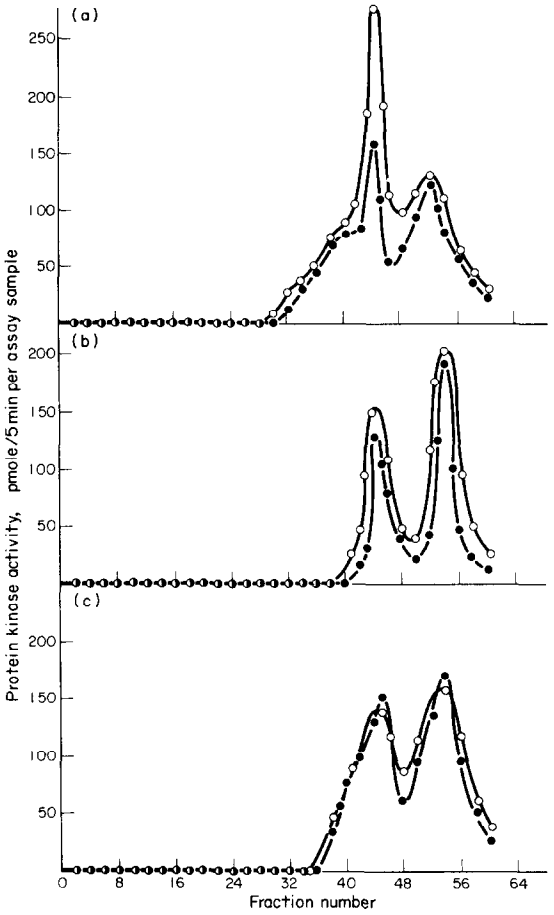

Fig. 2. Sephadex G-100 chromatography of Walker cell protein kinase 1.5 hr after the administration of solvent alone (A, control) or of chlorambucil (5 μg/ml) (B) or dibutyryl cAMP (100 μg/ml) (C).

lation is observed 1.5 hr after treatment and thereafter remains steady. This presumably reflects the time course for deacylation to N^6-monobutyryl cAMP, which will then cause an increase in the endogenous level of cAMP by inhibition of the low K_m form of the phosphodiesterase [19, 20].

The effect of chlorambucil on the cAMP-dependent protein kinase activity ratio in TLX5 lymphoma cells is shown in Table 1. These cells are more resistant

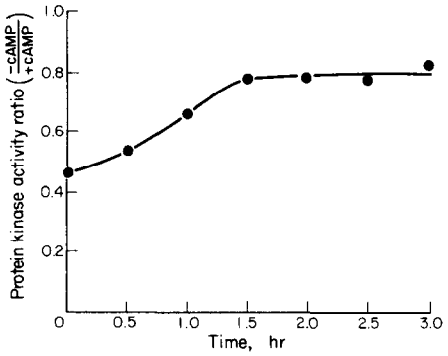

Fig. 3. Changes in the protein kinase activity ratio of sensitive Walker cells after treatment with 100 μg/ml of dibutyryl cAMP.

Table 1. Effect of chlorambucil (25 μg/ml) on the protein kinase activity ratio of TLX5 lymphoma

Time (hr)	−cAMP/+cAMP)	% of control
0	0.50	100
0.5	0.50	100
1.0	0.52	105
1.5	0.60	120
2.5	0.63	127
3.5	0.69	140
5.0	0.74	149
7.0	0.79	158

Table 2. Effect of chlorambucil concentration on the protein kinase activity ratio in sensitive (WS) and resistant (W_{CHL3}) Walker cells

Chlorambucil concentration (μg/ml)	Protein kinase activity ratio (−cAMP/+cAMP) Cell line	
	WS	W_{CHL3}
0	0.58	0.66
0.5	0.72	0.65
1.0	0.76	0.69
2.5	0.75	0.67
5.0	0.90	0.62
25.0	0.90	0.70
100.0	0.99	0.72

to chlorambucil (ID_{50} 1.0 μg/ml) than are Walker cells (Table 3), and a correspondingly higher dose of chlorambucil (25 μg/ml) was used. Again an increase in the protein kinase activity ratio is seen, but the time course of activation differs from that found in Walker cells with a steady increase in the ratio(−cAMP/+cAMP) over the time interval investigated (7 hr).

The effect of various doses of chlorambucil on the protein kinase activity ratio of sensitive and 200-fold resistant Walker cells (W_{CHL3}, Table 3) is shown in Table 2. For the sensitive line the ratio (−cAMP/+cAMP) increases with increasing concentrations of chlorambucil up to a maximum stimulation at 5 μg/ml, which corresponds to complete inhibition of cell growth. Further increases in the concentration of chlorambucil have little effect on the activity ratio.

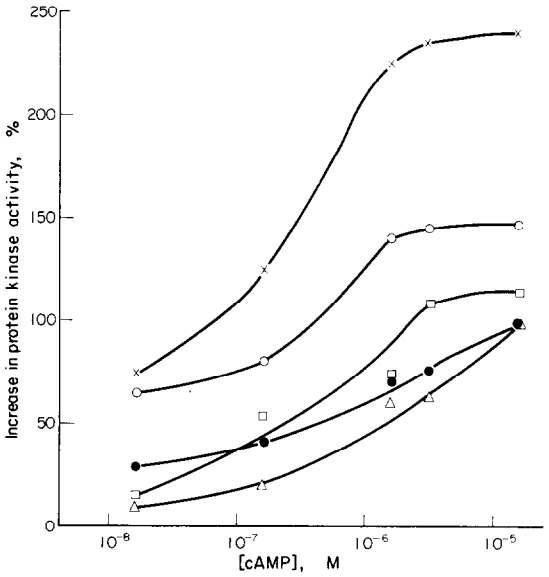

Fig. 4. Percentage stimulation of protein kinase activity of Walker cells by varying concentrations of cAMP, (×——×) sensitive cells; (○——○) W_{CHL1}; (●——●) W_{CHL2}; (△——△) W_{CHL3}; (□——□ W_{R4}).

In the resistant cell line, chlorambucil has no effect on the protein kinase activity ratio at low concentrations of the drug, and only a small stimulation is seen even at 100 μg/ml.

The degree of stimulation of the cAMP-dependent protein kinase in Walker cells resistant to either chlorambucil or to 5-aziridinyl-2,4-dinitrobenzamide (CB 1954) is much less than that observed in the sensitive line. This can be seen from the data in Fig. 4 which shows the amount of ^{32}P incorporated into histone by cytosolic protein kinases from sensitive and resistant Walker cells in the presence of increasing concentrations of cAMP. The ID_{50} values for the various cell lines towards chlorambucil and CB 1954, the concentration of cAMP needed to produce half-maximal stimulation of protein kinase, (K_a), for each of the cell lines and the specific activity of the enzyme for each cell line in the absence and in the presence of saturating concentrations of cAMP is shown in Table 3. Although the unstimulated kinase activity varies between the cell lines, the degree of stimulation

Table 3. Sensitivities of Walker cell lines to chlorambucil and CB 1954; Apparent K_a* values for activation of protein kinase and the specific activity of protein kinase measured in either the absence (−cAMP) or presence (+cAMP) of cAMP

Cell line	ID_{50} (μg/ml)			Sp. act. (pmole/min/mg protein)	
	Chlorambucil	CB 1954	$K_a \times 10^{-7}$ M	−cAMP	+cAMP
WS	0.045	0.0005	1.8	108	352
W_{CHL1}	1.9	0.002	3.0	164	396
W_{CHL2}	4.6	0.005	7.5	186	320
W_{CHL3}	9.0	0.008	20	231	381
W_{R1}	0.3	0.016	—	159	420
W_{R2}	1.5	0.03	—	256	312
W_{R4}	1.6	1.9	1.5	150	256
W_{R5}	2.0	8.0	—	146	247

* K_a values from the data presented in Fig. 4 is the concentration of cAMP producing half maximal stimulation of the protein kinase.

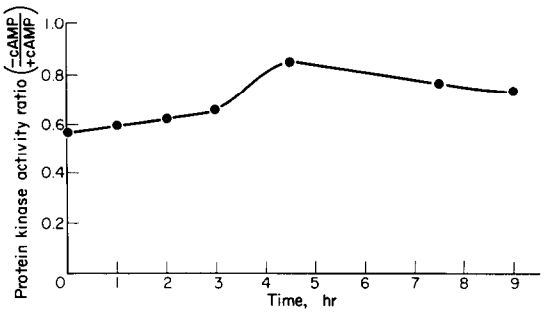

Fig. 5. Changes in the protein kinase activity in sensitive Walker cells after treatment with merophan (0.5 μg/ml).

by a given concentration of cAMP is always higher for the enzyme from sensitive cells, as is the maximum stimulation obtained at saturating concentrations of cAMP. It is of interest that the apparent K_a value for cAMP increases with increasing resistance. One cell line resistant to CB 1954 (W_{R2}) shows no stimulation of protein kinase activity by cAMP.

The effect of another bifunctional alkylating agent, merophan, on the protein kinase activity ratio of sensitive Walker cells is shown in Fig. 5. The dose of agent used (0.5 μg/ml) causes the same degree of growth inhibition (100 per cent) as does 5 μg/ml of chlorambucil. A similar increase in the protein kinase activity ratio is observed with a maximal inhibition occurring between 4 and 7 hr after treatment. This corresponds to the time at which the cAMP phosphodiesterase is maximally inhibited by this agent [4].

Although CB 1954 is a monofunctional alkylating agent, it shows a high therapeutic index against the Walker tumour [21], and growth inhibition by this agent can be prevented by 4-amino-2-phenylimidazole-5-carboxamide (2-phenyl-AIC) [22]. It has previously been shown [2] that CB 1954 causes an increase in the intracellular level of cAMP of Walker tumour 8 hr after treatment. The results presented in Fig. 6 show that CB 1954 (1 μg/ml) causes an increase in the protein kinase activity ratio in sensitive Walker cells, with a maximal effect being observed 6 hr after treatment. Addition of 2-phenyl AIC (100 μg/ml) to the cell suspension, 2 min before addition of 1 μg/ml of CB 1954 completely prevents the effect of the latter on protein kinase activity.

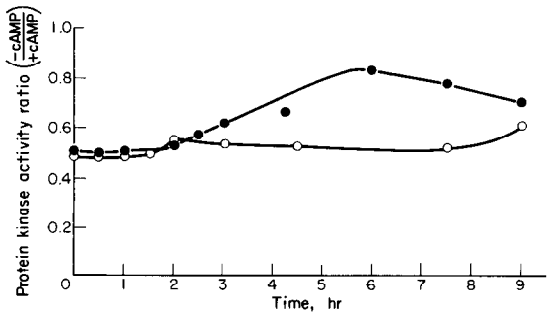

Fig. 6. Effect of 1 μg/ml CB 1954 (●——●) and 1 μg/ml CB 1954 plus 100 μg/ml 2-phenyl-AIC (○——○) on the protein kinase activity ratio of sensitive Walker cells.

DISCUSSION

A number of studies have shown an activation of cAMP-dependent protein kinases *in vivo* after treatment with agents which elevate the intracellular level of cAMP (13, 16, 17). Procedures for the determination of the state of activation of protein kinases in intact cells following hormone treatment have recently been described [14–16]. Measurement of cAMP-dependent protein kinase has been shown to be more reproducible than cAMP determinations [13].

The activity of cAMP-dependent protein kinase in the supernatant fraction of Walker cell homogenates measured in the absence of cAMP has been shown to be increased after administration of a therapeutic dose of chlorambucil. This rise in cAMP-dependent protein kinase occurs at the same time as the elevation of cAMP levels produced in these cells by chlorambucil [2] and at a time when the amount of cAMP-binding protein is decreased, as measured by competition with saturating concentrations of tritiated cAMP. The increase in the protein kinase activity ratio appears to be related to the concentration of chlorambucil and reaches a maximal activation at 5 μg/ml. Further increases in the concentration of chlorambucil have previously been shown to have no effect on the intracellular level of cAMP in sensitive Walker cells [4]. The results with Sephadex gel filtration indicate that the increase in protein kinase activity is not due to an artifact, and it is suggested that it reflects an activation of this enzyme *in vivo*. Furthermore, the degree of activation of the kinase with 5 μg/ml of chlorambucil is about equivalent to that obtained with 100 μg/ml of dibutyryl cAMP. These dose levels give 100 per cent inhibition of cell growth in sensitive Walker cells [8]. A similar activation of the cAMP-dependent protein kinase is seen in TLX5 cells at a higher dose of chlorambucil, and in Chinese hamster ovary cells and HeLa cells [23]. Two other effective anti-tumour alkylating agents, merophan and CB 1954, also cause an increase in the protein kinase activity ratio in sensitive Walker cells. In each case, maximal activation is seen at a dose corresponding to complete inhibition of cell growth.

The increase in the cAMP-dependent protein kinase activity ratio produced in Walker cells in response to CB 1954 is prevented by a dose of 2-phenyl-AIC which completely protects this tumour against the toxicity of CB 1954. This suggests that an increase in intracellular cAMP is important in the mechanism of action of CB 1954. The possibility that growth inhibition by the alkylating agents involves an initial rise in cAMP levels would also explain the apparently anomalous result that a monofunctional alkylating agent, which cannot cross-link DNA, is an active antitumour agent, and that when the Walker tumour becomes resistant to this drug it also becomes cross-resistant to bifunctional agents and vice-versa.

The cAMP-dependent protein kinase in a resistant Walker cell line is much less responsive to activation by chlorambucil *in vivo* and cAMP *in vitro*. A loss of cAMP-binding protein has previously been reported for Walker cells resistant to either chlorambucil or CB 1954 [8]. Granner [18] has shown an

altered regulation of cAMP-dependent protein kinase in a hepatoma cell line deficient in cAMP binding protein. The unstimulated protein kinase activity in this cell line was much higher than in liver. A similar situation exists for Walker cells where the unstimulated activity decreases in the order $W_{CHL3} > W_{CHL2} > W_{CHL1} \sim W_{R1} \sim W_{R4} \sim W_{R5} > WS$. Chlorambucil has previously been shown not to cause an appreciable increase in the cAMP levels of these cell lines [4]. The resistance of the protein kinase to cAMP stimulation cannot be ascribed to an increase in the cAMP phosphodiesterase activity since this has been shown to be reduced in the resistant cell lines [8].

Cyclic AMP-dependent protein kinase has been shown to phosphorylate both histone [9] and nonhistone chromosomal proteins [24] as well as enzymes [25] and microtubular proteins [26]. Any one of these substrates may be important for a cAMP-mediated event produced by the anti-tumour alkylating agents.

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