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ALTERATIONS IN ADENOSINE 3′,5′-MONOPHOSPHATE-BINDING PROTEIN IN WALKER CARCINOMA CELLS SENSITIVE OR RESISTANT TO ALKYLATING AGENTS

MICHAEL J. TISDALE and BARRY J. PHILLIPS

Department of Biochemistry, St. Thomas's Hospital Medical School, London SE1 7EH, England and
Ludwig Institute for Cancer Research, Chester Beatty Research Institute, Fulham Road, London SW3 6JB, England

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Abstract—The extent of binding of 8-[^{3}H]adenosine 3′,5′-monophosphate (cyclic AMP) to specific sites was measured in Walker carcinoma cells of tissue culture lines, having different degrees of resistance to the cytotoxic effects of alkylating agents. When compared with sensitive cells, the resistant lines showed a loss of binding activity with increasing resistance, when measured at either pH 4.0 or pH 6.5. This applied to cell lines made resistant either to 5-aziridinyl-2,4-dinitrobenzamide (CB 1954) or chlorambucil, although the total loss of binding activity was greater in the former cell lines. Scatchard analysis of binding suggested the presence of two sites in all cell lines with $K_{DI} \sim 1\text{–}5 \times 10^{-9}$ M and $K_{DII} \sim 3 \times 10^{-8}$ M. The decreased binding activity was not due to an increased cAMP phosphodiesterase activity in the resistant cell lines, since the activity of the high affinity form of this enzyme was either the same as (CB 1954-resistant) or less than (chlorambucil-resistant) that found in sensitive cells. Walker cells with acquired resistance to either CB 1954 or chlorambucil showed a degree of cross resistance to the cytostatic effect of cAMP analogues and of other alkylating agents.

Adenosine 3′5′-monophosphate (cyclic AMP), and agents which elevate the intracellular level of this nucleotide, cause tumour growth inhibition both *in vitro* [1–3] and *in vivo* [4–7]. One of the tumours which is highly susceptible to growth inhibition by cAMP is Walker 256 mammary carcinoma [4, 7]. It has been shown previously that the bifunctional alkylating agents cause an increase in the intracellular level of cAMP in Walker carcinoma cells, and that this increase is linearly related both to the concentration of the alkylating agent and to the cellular growth inhibition obtained [8, 9]. This increase in cAMP is thought to be due to an inhibition of the form of the cyclic nucleotide phosphodiesterase with a low K_m value, since only this form of the enzyme is inhibited and only in cells sensitive to the cytotoxic effect of the alkylating agents [8]. Resistance to bifunctional alkylating agents is accompanied by an alteration of this form of the enzyme expressed by a specific loss of two enzymic forms, a shift in pH optima and an altered inhibition constant to the reversible inhibitor theophylline [10]. While the bifunctional alkylating agent chlorambucil, which is an effective tumour growth inhibitor, causes an increase in intracellular cAMP in sensitive Walker carcinoma cells, the corresponding monofunctional analogue, which is ineffective as a tumour growth inhibitor, has no effect on cAMP levels, even at thirty-six times the dose of the bifunctional compound. However, the monofunctional aziridine derivative 5-aziridinyl-2,4-dinitrobenzamide (CB 1954), which is highly cytotoxic to Walker cells, produces an increase in cAMP which is comparable to that caused by chlorambucil at equitoxic doses [9]. Walker tumour cells with acquired resistance to CB 1954 also showed a degree of cross-resistance to chlorambucil and *vice versa*, implying a common mechanism of action for the two agents. However, the increase in cAMP produced in response to CB 1954 was shown not to be due to an inhibition of the cAMP phosphodiesterase. The cross-resistance might, therefore, involve a loss of responsiveness to the rise in cAMP level, which is an effect common to the two agents, rather than changes in cAMP synthesis or degradation.

Kuo and Greengard [11] have suggested that all the effects of cAMP might be mediated through the phosphorylation of specific proteins by protein kinases. Since binding of cAMP to a specific receptor appears to be an initial step in protein kinase activation, the degree of binding of cAMP to specific cytosolic proteins has been investigated in both sensitive Walker carcinoma cells and in those resistant to either chlorambucil or CB 1954.

MATERIALS AND METHODS

8-[^{3}H]Cyclic AMP (sp. act. 27.5 Ci/m-mole) was purchased from the Radiochemical Centre, Amersham. Cellulose ester filters were obtained from Millipore Corp., London, and N^6,$O^{2'}$-dibutyryl cAMP (db cyclic AMP) and N^6-monobutyryl cAMP (mb cyclic AMP) from Boehringer Corp., London. Scintillation fluid NE 233 was purchased from Nuclear Enterprises Ltd., Edinburgh. Chlorambucil and CB 1954 were synthesized at the Chester Beatty Research Institute, London.

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. A line of

Walker carcinoma cells, sensitive to alkylating agents, was established *in vitro*, as previously described [12]. One series of cell lines resistant to different concentrations of chlorambucil, and another series of CB 1954-resistant lines were developed from the sensitive line (WS) as follows. In the first instance, WS cells were suspended at 10^5/ml in fresh medium and dispensed into culture flasks. Chlorambucil or CB 1954 were made up at one hundred times the required concentration in DMSO and added to the cells to give a final concentration of 0.1 μg/ml of chlorambucil or 0.001 μg/ml of CB 1954. These concentrations were approximately twice the ID_{50} concentrations for WS cells (see below). The treated cells were incubated until a dense suspension was obtained. The suspension was then divided into two portions and diluted with medium to give 10^5 cells/ml. One portion was then treated again with the first dose, while the other received a 2-fold higher dose. This process was repeated so that some of the cells received regularly increasing doses of drug while others were diverted at each level and subsequently received the same drug dose every 10 days. In this way, a large number of lines was obtained. Three chlorambucil-resistant lines and four CB 1954-resistant lines were used in this study.

Drug sensitivity assays. The sensitivity of the various cell lines to drug treatment was estimated as described previously [12]. Cells were suspended in fresh medium at a concentration of 2×10^5/ml and the suspension was dispensed into tubes in 2-ml aliquots. Drug solutions were made up at one hundred times the required concentration and 20 μl were added to each tube. The cells were plated out, in the presence of drug, into the wells of a Linbro microtest plate, which holds 96 separate 200-μl samples. The plates were incubated and cell counts were made at 24-hr intervals. Growth curves were constructed from the counts and percentage inhibition of growth rate, compared with the control, was estimated. Dose–response curves were then constructed and ID_{50} values (dose of drug giving 50 per cent inhibition of growth) were read off.

Cyclic AMP-binding assay. The binding of cAMP to proteins was measured by a modification of the method of Gilman [13]. Cells were removed from tissue culture medium by centrifugation at 300 *g* for 3 min, after which they were washed with 0.9% NaCl and recentrifuged. All operations were then carried out at 0–4°. The cell pellets were suspended in 10 mM Tris–HCl, pH 7.6, containing 250 mM sucrose and 1 mM $MgCl_2$ and sonicated with a 20-Kc MSE sonic oscillator (5 sec/ml). Particulate material was removed by centrifugation at 100,000 *g* for 1 hr and the supernatant fraction was used for the determination of cAMP-binding activity. Protein was determined by the method of Lowry *et al.* [14] using bovine serum albumin as standard.

The cAMP-binding assay was performed in a final volume of 0.2 ml with either 50 mM sodium acetate, pH 4.0, or 50 mM potassium phosphate, pH 6.5, as buffers. For routine assays the buffers contained 3 mM theophylline and 80 nM 8-[^{3}H]cAMP (75,000 cpm per assay), and binding was allowed to take place for at least 2 hr at 0°. Cellular extracts were added in 0.1 ml of the Tris–sucrose sonicating buffer, usually at a protein concentration of 0.3–3 mg/ml (30–300 μg per assay). After the binding reaction had taken place for the desired length of time, 1 ml of cold 20 mM potassium phosphate buffer, pH 6.0, was added. After 2 min the contents of the tube were filtered by suction through a 25-mm cellulose ester filter (0.45 μm pore size) which had been premoistened with cold phosphate buffer. The tube was rinsed with 5 ml of cold phosphate buffer, which was also passed through the filter. Finally, the filter was rinsed with 10 ml of cold phosphate buffer. The filter containing protein-bound [^{3}H]cAMP was placed in a scintillation vial and dissolved in 1 ml of 2-(methoxy)ethanol. After dissolution, the amount of radioactivity was determined in 10 ml of scintillation fluid, [2-(methoxy)ethanol, 200 ml and 800 ml of a solution of 8 g of PPO in 1000 ml of toluene] using a Tracer Lab scintillation counter.

To determine the effect of pH on binding activity, the conditions were as described with 50 mM buffers (pH 3.6–5.7, sodium acetate and pH 7.0–8.0, Tris–HCl). The protein-bound cyclic AMP was assayed on Millipore filters with 20 mM buffers of the same pH.

Two types of corrections were used for non-specific binding. The 'filter blank' was obtained by measuring the amount of [^{3}H]cAMP bound to the filter in the absence of protein. A correction for non-specific cAMP-binding was made which was similar to that used by Daniel *et al.* [15]. In this case specific binding was calculated by subtracting the radioactivity retained by the filter in the presence of 1 mM unlabelled cAMP.

Assay of phosphodiesterase activity. The determination of phosphodiesterase activity has been previously described [16]. Incubations were carried out at 35° for a time interval which gave less than 10 per cent hydrolysis of the substrate. 8-[^{3}H]Cyclic AMP and 8-[^{3}H]5′AMP were separated by a thin-layer chromatographic procedure and radioactivity was determined in NE 233 scintillation fluid.

Cyclic AMP assay. Cyclic AMP was determined as previously described [8]. The ether extracted, lyophilized TCA supernatant was purified on a column of Dowex 50W-X8 200–400 mesh resin in the H^+ form, and the amount of cAMP in the eluate was determined using a Radiochemical Centre assay kit. A standard curve was performed for each determination.

RESULTS

Drug sensitivity of cell lines. The cell lines used in this work are listed in Table 1, along with their ID_{50} values for chlorambucil, CB 1954 and db cyclic AMP. WS is extremely sensitive to CB 1954 and considerably less so to chlorambucil. Comparatively large concentrations of db cyclic AMP are required to inhibit growth. The chlorambucil-resistant series W_{CHL} 1 to 3 had received their maximum dose as shown at least ten times before they were used. Despite this, none of the lines was unaffected by this dose, and their ID_{50} values were lower than might have been expected. This suggests that either survival after treatment was largely due to recovery over a longer period than was used in the sensitivity assay, or that resistant

Table 1. Cell lines used and their sensitivities to chlorambucil, CB 1954 and dibutyryl cyclic AMP

Cell line	Maximum dose previously survived (μg/ml)	ID_{50} μg/ml		
		Chlorambucil	CB 1954	db cAMP
WS	–	0.045	0.0005	32
	Chlorambucil			
W_{CHL1}	1.6	1.9	0.002	48
W_{CHL2}	6.4	4.6	0.005	100
W_{CHL3}	25	9.0	0.008	150
	CB 1954			
W_{R1}	0.016	0.3	0.016	57
W_{R2}	0.064	1.5	0.03	70
W_{R3}	0.5	1.4	0.35	50
W_{R4}	2.0	1.6	1.9	56

cells rapidly lost resistance after surviving each treatment. As shown in the table, these lines also showed a degree of resistance to CB 1954 and to db cyclic AMP, which increased with increasing chlorambucil resistance.

The ID_{50} values for the CB 1954 resistant lines W_R 1 to 4 show a closer correspondence with the concentrations with which the lines had repeatedly been treated. Cross-resistance to chlorambucil and db cyclic AMP were also shown by these lines.

Comparison of the activity of cyclic AMP-binding proteins in sensitive and resistant cells. The 100,000 *g* supernatant fractions from both sensitive and resistant Walker cells contain components which bind cAMP. The presence of phosphodiesterase in these fractions makes it impossible to measure binding at physiological pH except by using high concentrations of theophylline. This problem has been circumvented by using lower pH values at which to assess activity. Thus binding activity has been measured at both pH 6.5, which is close to physiological values, but at which the phosphodiesterase has minimal activity [10] and at pH 4.0 at which binding shows a maximum (Fig. 1). At both pH values the reaction was linear with protein concentrations between 30 and 300 μg per assay. The specific activity at these two pH values for both sensitive Walker cells and those resistant to either CB 1954 or chlorambucil is shown in Table 2. For all resistant cell lines and at both pH values, there is a loss of cAMP-binding protein. Since binding activity is proportionate to protein concentration and since both sensitive and resistant cells have similar protein contents (250 ± 10 μg/10^6 cells), it is assumed that the decreased binding activity in resistant cells represents a decreased concentration of binding protein per cell. This effect is most pronounced for Walker cells with acquired resistance to CB 1954 where there is a gradual loss of binding activity with increasing resistance, such that W_{R4} only has 30 per cent of the binding protein found in sensitive cells when measured at either pH value.

To discover if the lowering of the activity of specific cAMP binding in the resistant cells is due to the presence of an endogenous inhibitor, the effect of combinations on the binding activity has been measured. The results presented in Table 3 show some lowering of binding activity when sensitive and CB 1954 resistant cell cytosols are combined in equal proportions. There is little difference in binding activity at either pH value when sensitive and chlorambucil resistant cytosols are mixed. This suggests the possibility of an inhibitor to cAMP binding in CB 1954-resistant Walker cell cytosols.

When examining binding in unpurified extracts, the presence of endogenous cAMP must be considered. Such unlabelled cAMP would dilute the radioactive compound used to detect binding and might also mask some binding sites. The effect of dialysis on cAMP binding was therefore examined. The results presented in Table 4 show little difference in binding

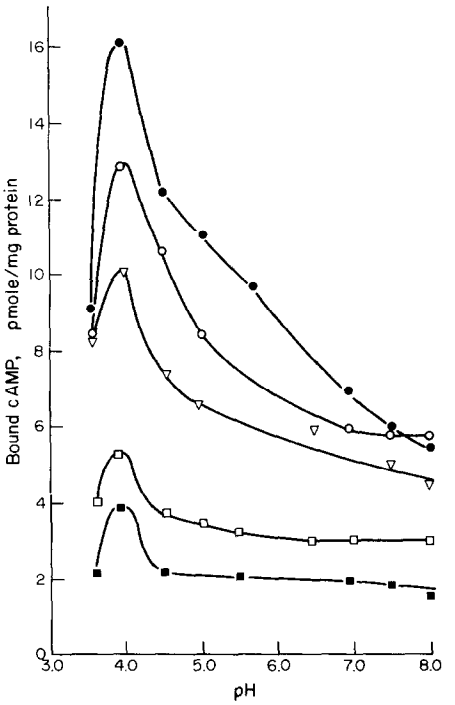

Fig. 1. The effect of pH on the binding of cAMP by cytosolic proteins from sensitive and resistant Walker cells. ●—● WS; ○—○ W_{CHL1}; ▽—▽ W_{CHL2}; □—□ W_{CHL3}; ■—■ W_{R2}.

Table 2. Specific activity of cyclic AMP-binding protein (pmole/mg protein) in Walker cells sensitive (WS) or resistant to either CB 1954 (W_R) or chlorambucil (W_{CHL})

Cell line	Binding activity (pmole/mg protein)	
	pH 4.0	pH 6.5
WS	13.3 ± 0.7	11.6 ± 0.6
W_{R1}	7.5 ± 0.4	6.3 ± 0.7
W_{R2}	4.7 ± 0.6	5.2 ± 0.6
W_{R3}	4.4 ± 0.7	3.9 ± 0.5
W_{R4}	4.2 ± 0.8	3.3 ± 0.1
W_{CHL1}	7.8 ± 0.5	8.5 ± 0.5
W_{CHL2}	7.5 ± 0.5	5.1 ± 0.4
W_{CHL3}	6.9 ± 0.6	6.7 ± 0.5

activity after 1 day's dialysis at either pH 4.0 or pH 6.5. After dialysis for 4 days, there is an appreciable loss of binding activity when measured at pH 4.0, but little difference at pH 6.5. Thus it appears that the endogenous cAMP makes little difference to the binding results as presented in Table 2. Differences in endogenous cAMP being responsible for the lowering of specific cAMP-binding activity in resistant cells can also be ruled out by the results presented in Table 5, which shows that the resistant cells either have the same or a lowered basal intracellular level of cAMP.

The possibility of quantitative differences in cAMP-binding proteins between sensitive and resistant cells was further investigated by an examination of the influence of pH on the binding of cAMP. The results presented in Fig. 1 show that binding by both sensitive and resistant cytosol has a pH optimum of about 4.0 at a ligand concentration of 100 nM. A similar pH optimum is shown for binding at 1 nM cAMP.

Comparison of phosphodiesterase activity in sensitive and resistant Walker cells. Since decreased binding of [^{3}H]cAMP could result from an increased rate of hydrolysis, the activity of cAMP phosphodiesterase has been measured in sensitive and resistant cells. This enzyme in both sensitive and resistant Walker cells has previously been shown to exist in two forms differing in K_m values; a low affinity form with $K_m \sim 80\,\mu M$ and a high affinity form with $K_m \sim 1.0\,\mu M$ [16]. The activity of this enzyme has therefore been determined at either 1 mM cAMP (which measured mainly the high K_m form of the enzyme) and at 5 μM cAMP (for the low K_m form).

Table 3. Effect of combinations of cytosolic protein from sensitive and resistant Walker carcinoma on the activity of the cyclic AMP binding protein at pH 4.0

Combination*	Binding activity (pmole/mg protein)	
	Observed	Expected
WS + W_{R1}	8.8 ± 0.5	10.4
WS + W_{R2}	7.4 ± 0.4	9.0
WS + W_{R3}	7.3 ± 0.4	8.8
WS + W_{R4}	6.9 ± 0.6	8.7
WS + W_{CHL1}	12.1 ± 0.7	9.7
WS + W_{CHL2}	11.5 + 0.5	10.4
WS + W_{CHL3}	11.7 ± 0.6	11.5

* Cytosolic fractions of sensitive and resistant cells were mixed in equal proportions.

Table 4. Effect of dialysis on the cyclic AMP-binding protein of sensitive Walker carcinoma

		Time (days)		
	pH	0	1	4
Activity (pmole/mg protein)	4.0	13.8	13.4	5.2
	6.5	11.6	13.6	10.5

The specific activity of the enzyme expressed as nmoles of 5'AMP formed/min/milligram of protein in total cell sonicated suspensions of sensitive and resistant cells is shown in Table 6. When assayed at 1 mM cAMP, the specific activity of the enzyme is approximately constant in both sensitive and resistant cells within the limits of experimental error. However, when assayed at low substrate concentration there is a gradual decrease in the specific activity of the low K_m form of the enzyme with increasing resistance to chlorambucil. A similar loss of this form of the enzyme has previously been found in cells resistant to melphalan [8, 16]. For Walker cells with acquired resistance to CB 1954, there is no loss of the high affinity form of the phosphodiesterase with increasing resistance. Since the activity of the cAMP phosphodiesterase in resistant Walker cells is either the same or lower than that of the sensitive cells, decreased availability of the added [^{3}H]cAMP does not account for the differences in binding protein activity.

Comparison of Scatchard plots for cyclic AMP binding. To compare the binding components in sensitive and resistant cells more thoroughly, the amount of [^{3}H]cAMP bound has been measured as a function of total ligand concentration and the data has been used to construct Scatchard plots [17]. A wide range of cAMP concentrations was employed in order to look for potential heterogeneity in the binding. The Scatchard plots shown in Fig. 2 can be interpreted as showing two non-interacting binding sites in both sensitive and resistant Walker cells with $K_{DI} \sim 1\text{–}5 \times 10^{-9}$ M and $K_{DII} \sim 3 \times 10^{-8}$ M. The dissociation constants for both sensitive and resistant cells at pH 6.5 are shown in Table 7. In all cell types, the high affinity binding site is quantitatively the most important. Only W_{CHL3} cells lack the low affinity binding site (Fig. 2). The results could equally be interpreted as being one site showing negative cooperativity. The specific binding of cAMP by cytosolic fractions from sensitive and resistant Walker cells at various cAMP concentrations is shown in Fig. 3. This indicates that there is a gradual loss of binding activity at pH 6.5 with increasing resistance at all cAMP

Table 5. Cyclic AMP content of sensitive and resistant Walker cells

Cell line	Cyclic AMP (pmole/mg protein)
WS	42 ± 2.0
W_{CHL1}	40 ± 2.0
W_{CHL2}	37 ± 2.0
W_{CHL3}	34 ± 2.0
W_{R1}	43 ± 2.0
W_{R2}	42 ± 2.0
W_{R3}	43 ± 2.0
W_{R4}	51 ± 2.0

Table 6. Cyclic AMP phosphodiesterase activities of sensitive and resistant Walker carcinoma

Cell line	Cyclic AMP phosphodiesterase activity (nmole/min/mg protein)	
	High K_m activity*	Low K_m activity†
WS	1.68 ± 0.05	0.26 ± 0.01
W_{R1}	1.66 ± 0.05	0.25 ± 0.03
W_{R2}	1.70 ± 0.05	0.25 ± 0.05
W_{R3}	1.61 ± 0.05	0.24 ± 0.02
W_{R4}	1.69 ± 0.05	0.255 ± 0.005
W_{CHL1}	1.67 ± 0.05	0.22 ± 0.004
W_{CHL2}	1.65 ± 0.05	0.14 ± 0.02
W_{CHL3}	1.68 ± 0.05	0.094 ± 0.001

* Measured at 1 mM cAMP.
† Measured at 5 μM cAMP.

concentrations greater than ~10 nM, such that WS > W_{CHL1} > W_{CHL2} > W_{R1} > W_{CHL3} > W_{R2} > W_{R3}. A similar loss of binding activity with increasing resistance was also observed at pH 4.0. These results therefore suggest that resistant cells contain fewer cAMP receptors than do sensitive cells.

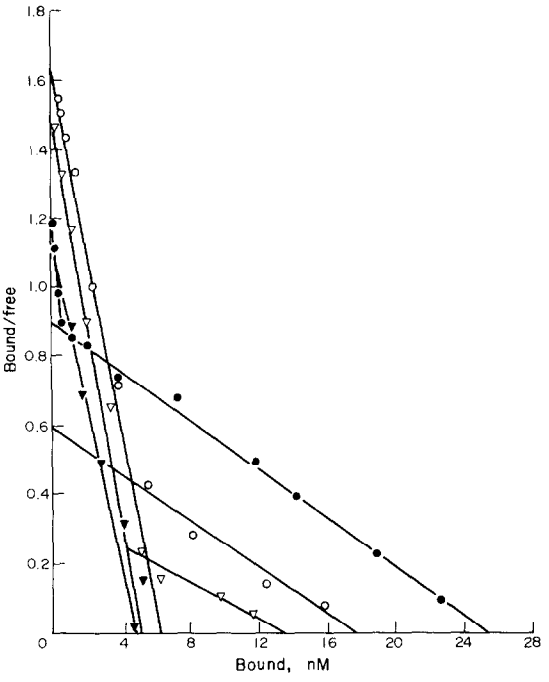

Fig. 2. Scatchard plots for the binding of cAMP by cytosolic proteins from sensitive and resistant Walker cells shown in Fig. 3. ●——● WS; ○——○ W_{CHL1}; ▽——▽ W_{CHL2}; ▼——▼ W_{CHL3}.

DISCUSSION

Cyclic AMP is believed to activate cAMP-dependent protein kinases by binding to the regulatory subunit of the inactive holoenzyme form (RC) causing a dissociation into the regulatory subunit-cAMP complex (R. cAMP) and the active catalytic subunit (C) [18–20]. Dissociation constants ranging from 0.5 nM [21] to 110 μM [22] have been reported for the binding of cAMP to RC. The present results with Walker carcinoma cells sensitive and resistant to alkylating agents suggest at least two types of cytosolic cAMP-binding sites as revealed by Scatchard analysis; a high affinity site with $K_D \sim 1\text{–}5 \times 10^{-9}$ M and a low affinity one with $K_D \sim 3 \times 10^{-8}$ M, the former being quantitatively the most important.

Daniel, Litwack and Tomkins [15] have shown that mouse lymphosarcoma cells resistant to the cytotoxic effect of db cyclic AMP contain less cytoplasmic cAMP binding proteins and decreased cAMP stimulated protein kinase activity compared with the sensitive line. They suggested that cytosol from the sensitive cells might contain two types of cAMP binding sites and that with the acquisition of resistance, there was a loss of the lower affinity sites and a decreased concentration of the higher affinity sites. We have shown a similar decrease in the specific cAMP binding sites in Walker carcinoma with acquired resistance to both the monofunctional alkylating agent, CB 1954, and also to the difunctional agent, chlorambucil, although the loss of binding activity is more pronounced in the former cell lines. These cells also show a degree of cross-resistance to db cyclic AMP. However, W_{R4} which requires 3600 times as much CB 1954 as WS does to give 50 per cent growth inhibition, only requires three times as much db cAMP. This is surprising in view of the 70 per cent loss of binding protein in this cell line.

Cho-Chung has separated two cell populations of Walker carcinoma, one responsive and the other unresponsive to db cyclic AMP [23]. Both tumours respond to treatment with a doubling of cAMP levels [4]. The unresponsiveness to cAMP in the resistant tumour has been correlated with an altered cAMP binding by the tumour cytosol [24]. The resistant tumour has only 50 per cent of the specific cAMP-binding activity of the sensitive line and a higher K_D for cAMP binding. This particular line of the Walker tumour which is sensitive to cAMP only possesses the high affinity binding sites, while the unresponsive tumour only has the low affinity sites.

It has been reported previously [10] that Walker cells resistant to the bifunctional alkylating agent, nelphalan, show an alteration of the cAMP phosphodiesterase, which is manifested by a change in pH optimum and a decreased contribution of the high affinity form of the enzyme to the total activity. This loss of the high affinity form of the phosphodiesterase with increasing resistance is also seen in Walker cells resistant to chlorambucil. There is not, however, a loss of this form of the enzyme in cells resistant to

Table 7. Dissociation constants K_D of cyclic AMP binding to cytosolic proteins of sensitive and resistant Walker cells at pH 6.5

Cell line	$K_{D1} \times 10^{-9}$ M	$K_{D11} \times 10^{-8}$ M
WS	1.3 ± 0.5	3.1 ± 0.5
W_{R1}	3.9 ± 0.4	3.1 ± 0.5
W_{R2}	1.6 ± 0.5	3.9 ± 0.4
W_{R3}	1.5 ± 0.5	3.5 ± 0.4
W_{R4}	2.0 ± 0.4	5.6 ± 0.6
W_{CHL1}	4.6 ± 0.4	3.0 ± 0.7
W_{CHL2}	3.9 ± 0.6	3.6 ± 0.4
W_{CHL3}	5.0 ± 0.6	—

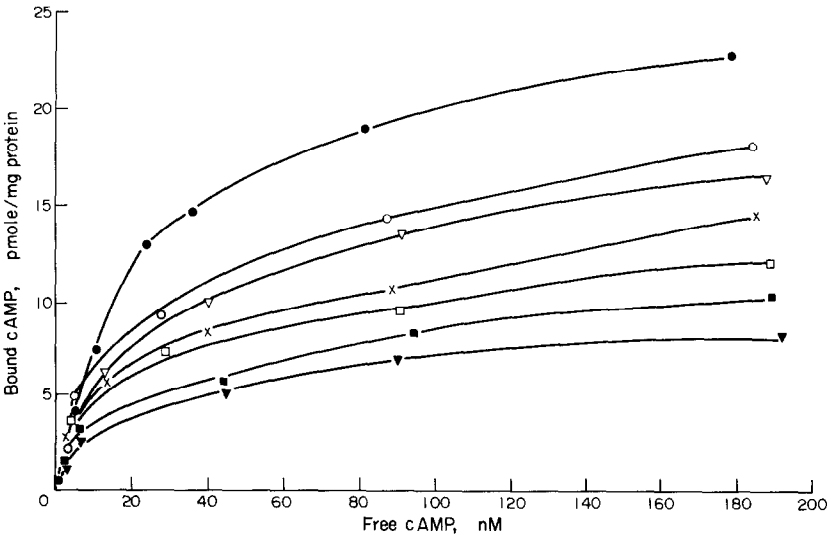

Fig. 3. Specific cAMP binding by cytoplasmic extracts from sensitive and resistant Walker cells at various cAMP concentrations. ●—● WS; ○—○ W_{CHL1}; ▽—▽ W_{CHL2}; □—□ W_{CHL3}; ×—× W_{R1}; ■—■ W_{R2}; ▼—▼ W_{R3}.

CB 1954. This is in accord with the observation that chlorambucil causes an elevation of intracellular cAMP by inhibiting the phosphodiesterase with a low K_m value, whilst CB 1954 has no effect on the activity of this enzyme at a time when cAMP levels are elevated [8].

Despite an apparent difference in the mechanism by which the two drugs cause an elevation of cAMP, loss of binding activity could explain the cross-resistance observed between chlorambucil and CB 1954, assuming that binding is important in the manifestation of the effects of cAMP.

Daniel *et al.* [25] found a lower level of phosphodiesterase activity in mutant mouse lymphoma cells resistant to db cAMP, and postulated that the lower level of enzyme activity was related to a deficiency of cAMP binding activity. In the present investigation, although a loss of both binding activity and of the high affinity form of the phosphodiesterase was observed in Walker cells resistant to chlorambucil, no loss of this form of the enzyme was observed in Walker cells resistant to CB 1954 although they have a much greater loss of binding activity.

Since the presently identified function of the cAMP receptor is the regulation of cAMP-dependent phosphokinase, variations in the stimulation of this enzyme in Walker cells sensitive and resistant to alkylating agents are currently under study.

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