EFFECT OF DIBUTYRYL ADENOSINE CYCLIC 3′,5′-MONOPHOSPHATE AND TESTOLOLACTONE ON CONCANAVALIN A BINDING AND CELL KILLING

BRIAN STORRIE. From the Eleanor Roosevelt Institute for Cancer Research, Department of Biophysics and Genetics, University of Colorado Medical Center, Denver, Colorado 80220

INTRODUCTION

Pulse exposure of Chinese hamster ovary cells in vitro to the lectin, concanavalin A (Con A), causes preferential rounding of the epithelial (E) form of these cells (1, 2). The fibroblast (F) form, produced upon growth of cultures in the presence of reverse transformation agents such as dibutyryl cyclic AMP (DBcAMP) supplemented with testosterone derivatives or certain prostaglandins (1, 3), is resistant to rounding during brief exposure to Con A. However, with prolonged exposure to Con A, either cell population rounds completely (2). The time lag in Con A rounding of cells treated with DBcAMP plus testosterone suggests that cell rounding may be an autocatalytic or cooperative process. The conversion of cells to Con A resistance is rapid, reversible, and insensitive to inhibitors of RNA and protein synthesis or of microtubule formation and caused by a synergistic interaction between DBcAMP and testosterone (2).

In the present work, the effect of treatment of Chinese hamster ovary cells with DBcAMP plus testololactone, a testosterone derivative, on Con A binding and cell killing and the effect of respiratory inhibitors on Con A cell rounding have been investigated. Con A cell rounding has been found to be sensitive to low temperature and inhibitors of respiration. Cells in either the absence or presence of DBcAMP plus testololactone were found to be equally sensitive to Con A killing and to bind equal amounts of [^{125}I]Con A.

MATERIALS AND METHODS

Cells were cultured as described previously (2). In the current experiments testololactone (4) is used instead of the previously used testosterone propionate in order to avoid possible toxic effects of the latter compound. The growth rate of cultures plated in the absence or presence of the reverse transformation agents, 0.3 mM DBcAMP plus 3.3×10^{-5} M Δ-1-testololactone, is identical for the first 24 h after plating. To determine the effect of various agents upon cloning efficiency, cells were plated in duplicate, exposed to the agent, and clones counted after 6 days of growth. The cloning efficiency of control cells was ~80%.

Cultures were incubated at 37°C with Con A in a humidified CO_2 incubator (unless otherwise indicated) and rounded cells scored as previously described (2). In experiments to test the effect of temperature on Con A cell rounding, cultures were preincubated for 5 min with 2°C medium minus or plus DBcAMP and testololactone and with 20 mM HEPES buffer (pH 7.2) added to control medium pH outside the CO_2 incubator. Cultures

were then incubated with Con A at 2°C for various times. The addition of HEPES buffer was without effect on Con A cell rounding. In energy inhibition experiments, cultures were pretreated for 30 min with inhibitors of energy metabolism before Con A addition.

Specific iodination of tyrosine residues by lactoperoxidase (Calbiochem, San Diego, Calif.) at 22°C was carried out by slight modifications of the procedure of Arndt-Jovin and Berg (5). Con A at a concentration of 1×10^{-4} M was dissolved in phosphate-buffered saline (PBS; reference 6), containing 0.1 M α-methylmannopyranoside and 1×10^{-3} M [^{125}I]Na (specific activity, 450–650 μCi/μmol). The reaction was initiated by the addition of lactoperoxidase (7.7×10^{-7} M) and H_2O_2 (1×10^{-4} M) and stopped after 20 min by dialysis at 4°C against PBS, 0.1 M sucrose for 16–18 h with three changes, followed by dialysis against 400 vol PBS for 1 h in three changes to remove sucrose. The ^{125}I-labeled Con A was then used immediately. The specific activity of the preparations varied between 4,700–6,300 cpm/μg.

Cells were treated with [^{125}I]Con A in F12 medium with 5% dialyzed calf serum minus or plus DBcAMP and testololactone as appropriate either at 37°C followed by incubation in a humidified CO_2 incubator or at 2°C as described above. Treatment was terminated by removal of medium followed by four washes with 2°C PBS. After solubilization of the cultures in 0.5 N NaOH, radioactivity was analyzed by liquid scintillation spectrometry. Parallel petri dishes without cell inoculum were incubated with [^{125}I]Con A for each experimental determination to control for possible binding of Con A to serum proteins attached to the dish (7, 8). Specificity of [^{125}I]Con A binding was tested by pretreating cultures with α-methylmannopyranoside (9) at a concentration of 0.05 M.

In control experiments, little if any difference in [^{125}I]Con A bound was found with between three and five washes with either 2°C PBS or with warm medium minus or plus reverse transformation agents. Phosphate-buffered saline washes were found to cause detachment of only 3–5% of the cell population.

The volume of cells grown in the absence or presence of reverse transformation agents was estimated by removing the cells from the petri dishes by trypsin and determining the relative cell volume with a Coulter counter (Coulter Electronics, Inc., Hialeah, Fla.). The lower threshold value of the counter aperature giving 50% of the maximum cell count was considered to be the average cell volume. The surface area was then calculated assuming the cells in suspension to be perfect spheres.

RESULTS

Killing of Cells Grown in the Absence or Presence of Reverse Transformation Agents

The ultimately complete rounding by Con A of cells grown in the absence or presence of DBcAMP plus testosterone (2) suggests that cells grown in the absence or presence of reverse transformation agents may be equally susceptible to Con A killing.

Fig. 1 shows the effect of Con A on the cloning efficiency of Chinese hamster ovary-K1 cells grown in the absence (E form) or presence (F form) of reverse transformation agents. Continuous exposure of either cell form to ≤ 5 μg/ml Con A causes little to no reduction in cloning efficiency or in colony size. 10 μg/ml Con A causes a reduction in cloning efficiency and reduces the colony size of cultures in basal medium (E form) by ~75%. Little reduction of F form colony size

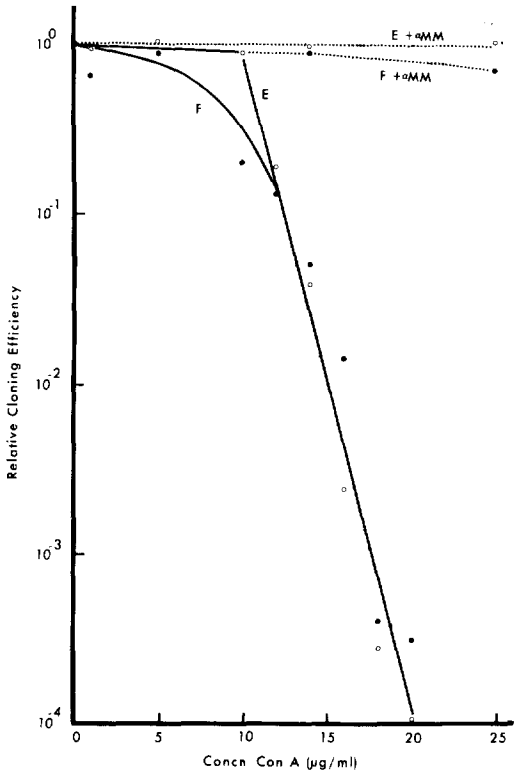

FIGURE 1 Effect of Con A on the cloning efficiency of cells grown in the absence (E or epithelial form) or presence (F or fibroblast form) of DBcAMP plus testololactone. Cells were plated in the absence or presence of reverse transformation agents. 18–20 h post-plating, Con A was added to the preexisting medium. α-methylmannopyranoside (αMM) at a final concentration of 0.01 M was added to some cultures. O—O Relative cloning efficiency of E form cells in the absence of αMM. ●—● Relative cloning efficiency of F form cells in the absence of αMM. O- - - -O Relative cloning efficiency of E form cells in the presence of αMM. ●- - - -● Relative cloning efficiency of F form cells in the presence of αMM.

occurs at this concentration. With > 10 μg/ml Con A the cloning efficiency of cells in either the absence or presence of DBcAMP plus testololactone is decreased equally and precipitously. No colonies of either cell form have been observed in cultures (2×10^6 cells) exposed continuously to 40 μg/ml Con A. Addition of 0.01 M α-methylmannopyranoside (αMM), a specific inhibitor of Con A binding (9), prevents Con A inhibition of colony formation.

To test whether Con A growth inhibition results in actual cell death, the reversibility of Con A inhibition of colony formation was examined. 1-day exposure to 40 μg/ml Con A causes an appreciable decrease in cloning efficiency of E or F form cells, and by the end of 3-days' exposure to Con A no colonies are found 6 days after Con A removal (Table I). In previous work, a 6-h exposure to 40–100 μg/ml Con A was shown to cause no reduction in cloning efficiency (2).

Concentration Dependence and Kinetics of [^{125}I]Con A Binding to Cells Grown in the Absence or Presence of Reverse Transformation Agents

To test whether the difference between E and F form cells in Con A rounding in short term experiments is due a difference in the amount of Con A bound to either cell form, the concentration dependence and kinetics of binding of ^{125}I-labeled Con A to cells was investigated.

Fig. 2 shows the concentration dependence and kinetics of Con A binding to E or F form cells during incubation with [^{125}I]Con A. No difference is apparent in the amount of [^{125}I]Con A bound in a 30 min or 2 h exposure to cells grown in the absence (E) or presence (F) of reverse transformation agents at any concentration between 0.5 and 100 μg/ml (Fig. 2). In control experiments, ^{125}I-labeled Con A was found to have normal biological activity as previously reported by others (5, 10, 11). About two and one-half times as much Con A is bound per cell after a 2-h exposure to [^{125}I]Con A than in a 30-min incubation.[1] Under the binding conditions used, only 3–5%, at most, of the apparent binding is not blocked by 0.05 M α-methylmannopyranoside. Fig. 2 shows the kinetics of binding of [^{125}I]Con A at a concentration of 40 μg/ml, a concentration of Con A showing great differences in rounding between E and F form cells (2). No significant difference in Con A binding at 37°C to E or F form cells is found at any time point between 5 min and 2 h.

TABLE I

Reversibility of Con A Inhibition of Cell Growth

Con A exposure (40 μg/ml)	Cloning efficiency	
	E	F
Control	85 %	82 %
1 day	34 %	11 %
2 days	1 %	< 0.5%
3 days	< 0.5%	< 0.5%

200 cells were plated in basal medium in the absence (E or epithelial form) or presence (F or fibroblast form) of DBcAMP plus testololactone. 18-h post-plating, Con A was added to the preexisting medium. At various times, Con A-containing medium was removed and replaced with Con A-free medium.

A portion of Con A binding at 37°C may reflect pinocytotic or other forms of uptake and may mask important differences in [^{125}I]Con A binding to the cell surface. In fact, E and F form cells pretreated with 2°C medium bind equal amounts of Con A in a 5-min to 2-h exposure to 40 μg/ml [^{125}I]Con A at 2°C, a temperature at which pinocytosis is blocked (Fig. 3). At 2°C neither cells grown in the absence or presence of reverse transformation agents are rounded above background during a 2-h exposure to 40 μg/ml of Con A. Replacement of the cold medium with warm, Con A-free medium results in a significant rounding of the cells during the ensuing 2-h period.

Only slight differences in the amount of Con A bound per unit of cell surface may exist. Little difference in cell size is found between E and F form cells detached from substrate. The volume of F form cells is 13% larger than that of E cells, and the surface area is 9% larger assuming each cell form in suspension to be a perfect sphere.

Effect of Inhibitors of Respiration on Con A Cell Rounding

The inhibition of Con A cell rounding by low temperature may indicate a possible dependence of rounding on cellular respiration. To examine this possibility, cells were pretreated for 30 min with inhibitors of glycolysis or oxidative phosphoryla-

[1] The lack of saturation in these experiments is apparently due to the presence of calf serum. Con A by binding serum components may form a multilayered lattice on the cell surface.

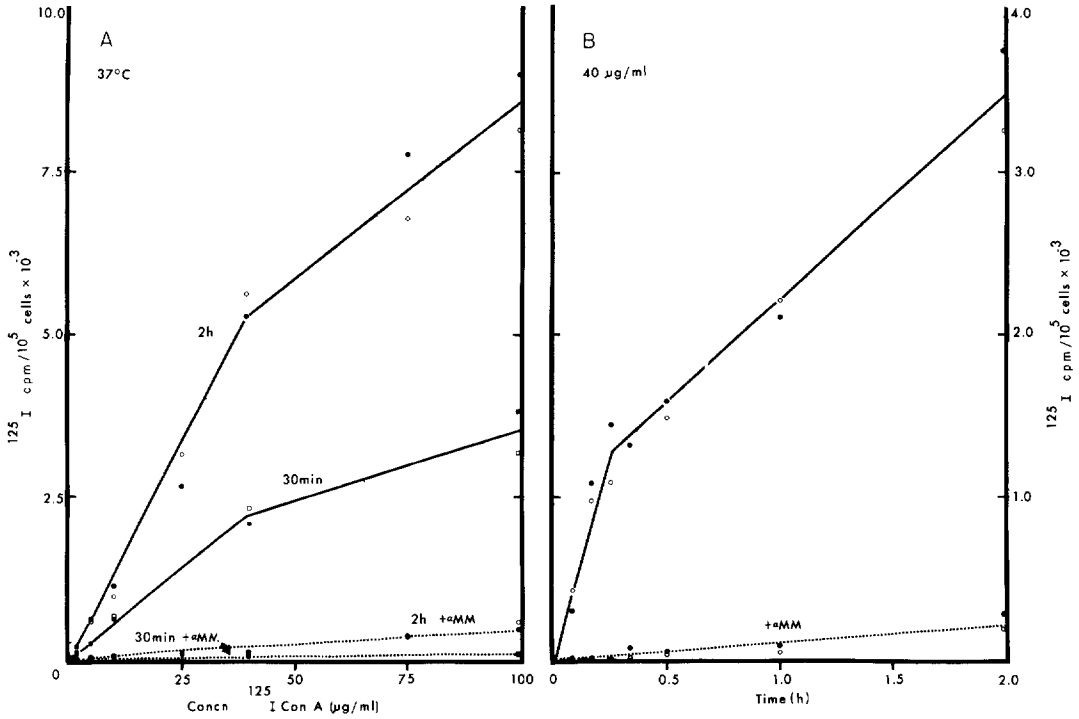

FIGURE 2 Concentration (A) and time (B) dependence of [^{125}I]Con A binding at 37°C to Chinese hamster ovary-K1 cells grown in the absence (E or epithelial form) or presence (F or fibroblast form) of DBcAMP plus testololactone for 18 h. In (A) □—□, ■—■ cpm [^{125}I]Con A bound during 30-min treatment of E or F form cells, respectively, in the absence of 0.05 M α-methylmannopyranoside (αMM). □- - - -□, ■- - - -■ cpm [^{125}I]Con A bound during 30-min treatment of E or F form cells, respectively, in the presence of 0.05 M αMM. ○, ● cpm [^{125}I]Con A bound during 2-h treatment of E or F form cells respectively, in the absence or presence of 0.5 M αMM. In (B) ○—○, ●—● cpm bound during treatment of E or F form cells, respectively, with 40 μg/ml [^{125}I]Con A in the absence of 0.05 M αMM. ○- - - -○, ●- - - -● cpm bound during treatment of E or F form cells, respectively, with 40 μg/ml [^{125}I]Con A in the presence of 0.05 M αMM.

tion and then exposed to Con A in the presence of inhibitor.

Treatment of cells in the absence (E) or presence (F) of reverse transformation agents with inhibitors of either glycolysis or oxidative phosphorylation significantly depresses Con A cell rounding (Table II). The F form appears to be more sensitive to energy inhibition than the E form. Use of inhibitors of glycolysis and oxidative phosphorylation together gives most effective inhibition of Con A rounding. If allowance is made for the background of ~6% rounded cells in non-Con A-treated cultures, cyanide or azide together with sodium fluoride gives ~80% inhibition of Con A rounding of E form cells and almost 100% inhibition of F form rounding. A similar synergistic inhibition of rounding by inhibitors of glycolysis and oxidative phosphorylation together can be observed using 1×10^{-5} M iodoacetate instead of sodium fluoride.

The effect of respiratory inhibitors on Con A rounding is not the result of binding of insufficient amounts of Con A. Replacement of the inhibitor (1×10^{-3} M KCN, 2×10^{-3} M NaF) Con A-containing medium with warm Con A-free medium results in rounding of either cell form to the same extent as the control cultures treated with Con A in the absence of inhibitors for 2 h.

DISCUSSION

The equal inhibition of colony formation by Con A of Chinese hamster ovary-K1 cells grown in either the absence (epithelial or E form) or presence (fibroblast or F form) of DBcAMP plus testololactone is consistent with the previously reported ability of Con A to round completely either cell

form over a period of hours (2). That Con A actually kills cells is demonstrated by the nonreversible inhibition of colony formation occurring during a 1 to 3-day exposure to 40 μg/ml Con A.

The almost complete rounding of cells grown in the absence or presence of reverse transformation agents during a 6-h Con A treatment (2) and the equal susceptibility of either cell form to Con A killing suggests, but does not prove, that equal numbers and kinds of Con A receptors may be present on the surface of either cell form. Binding experiments with [^{125}I]Con A at 2° or 37°C show no difference in the amount of Con A associated with E or F form cells at any concentration between 0.5 and 100 μg/ml or at any exposure between 5 min and 2 h. The difference in rounding response of cells grown in the absence or presence of DBcAMP plus testololactone cannot be explained on the basis of a difference in the amount of Con A bound per cell. Since there is little difference in cell size between the two cell forms in suspension, little if any difference in the amount of Con A bound per unit of cell surface exists.

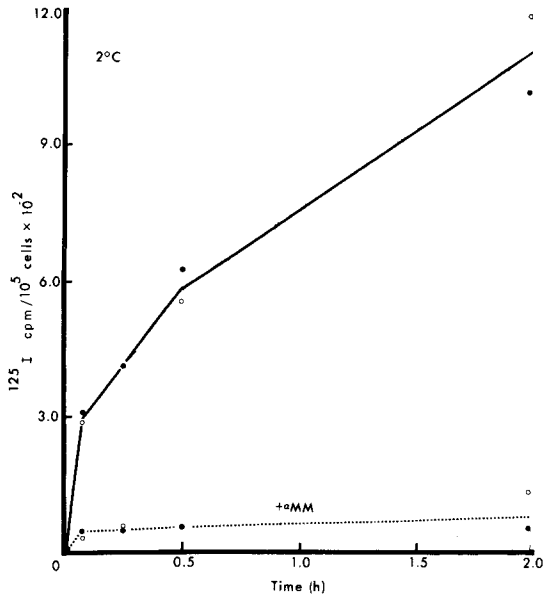

FIGURE 3 Kinetics of binding at 2°C of 40 μg/ml [^{125}I]Con A to cells grown in the absence (E or epithelial form) or presence (F or fibroblast form) of DBcAMP plus testololactone for 17 h. O—O, ●—● cpm [^{125}I]Con A bound to E or F form cells, respectively, in the absence of 0.05 M α-methylmannopyranoside (αMM). O- - - - -O, ●- - - - -● cpm [^{125}I]Con A bound to E or F form cells, respectively, in the presence of 0.05 M αMM.

TABLE II

Effect of Inhibitors of Respiration on Con A Cell Rounding

Inhibitor	Frequency of cells rounded by 2-h challenge with 40 μg/ml Con A	
	E	F
None	83%	44%
Glycolysis		
2×10^{-3} M sodium fluoride	77%	27%
2×10^{-2} M sodium fluoride	28%	16%
5×10^{-4} M iodoacetate	38%	7%
5×10^{-3} M iodoacetate	22%	9%
Oxidative phosphorylation		
1×10^{-3} M potassium cyanide	57%	6%
1×10^{-2} M sodium azide	48%	11%
Glycolysis plus oxidative phosphorylation		
1×10^{-3} M potassium cyanide, 2×10^{-3} M sodium fluoride	23%	5%
1×10^{-2} M sodium azide, 2×10^{-3} M sodium fluoride	27%	6%

Cells were plated in basal medium in the absence (E or epithelial form) or presence (F or fibroblast form) of DBcAMP plus testololactone. 17-h post-plating cells were pretreated for 30 min with inhibitor(s) and then exposed to Con A in the presence of inhibitor(s).

A similar amount of Con A has been reported to bind to the surface of normal and transformed cells by other authors (5, 11, 12). However, Noonan and Burger (13) have reported recently that transformed cells bind three and one-half to five times more [^{3}H]Con A than do normal cells during a 5-min exposure to [^{3}H]acetyl- or succinyl-Con A at 0°C.

If Con A rounding is due directly to binding to the cell surface and possible interactions of Con A molecules themselves, the temperature dependence and inhibition of rounding by respiratory inhibitors suggest that Con A cell rounding is mechanistically more similar to ligand-induced cap formation in lymphocytes than to a cell agglutination reaction. Capping is both temperature and respiration dependent (14–16). Agglutination, while temperature dependent, is not respiration dependent (16). Electron microscope observations suggest that Con A may induce cap formation in Chinese hamster ovary-K1 cells, and experiments with fluoroscein-conjugated Con A demonstrate a close correlation between capping and rounding (Storrie, manuscript in preparation). Cyclic AMP treatment of Chinese hamster ovary-K1 cells may cause

Con A binding sites to be more firmly fixed in place in the cell membrane and hence restrict ligand-induced movements.

The possibility that the mode of action of Con A in causing cell rounding may be a more indirect, secondary effect of Con A upon the internal concentration of a small molecule, such as cAMP or cGMP or some other process, cannot presently be excluded and is currently being investigated.

I would like to express my appreciation to Dr. Theodore T. Puck for encouragement, support, and critical reading of this manuscript, and to Dr. David Patterson for helpful discussions.

This work was aided by an American Cancer Society Grant No. VC-81B, and by a postdoctoral fellowship from the American Cancer Society (PF-899).

This publication is No. 571 from the Department of Biophysics and Genetics, University of Colorado Medical Center, Denver, Colorado.

Received for publication 16 November 1973, and in revised form 21 February 1974.

REFERENCES

1. HSIE, A. W., C. JONES, and T. T. PUCK. 1971. *Proc. Natl. Acad. Sci. U. S. A.* **68**:1648.
2. STORRIE, B. 1973. *J. Cell Biol.* **59**:471.
3. HSIE, A. W., and T. T. PUCK. 1971. *Proc. Natl. Acad. Sci. U. S. A.* **68**:358.
4. PUCK, T. T., and L. WENGER. 1973. *IRCS Int. Res. Commun. System.* June 1973.
5. ARNDT-JOVIN, D. J., and P. BERG. 1971. *J. Virol.* **8**:716.
6. DULBECCO, R., and M. VOGT. 1954. *J. Exp. Med.* **99**:167.
7. SMITH, S. B., and J.-P. REVEL. 1972. *Dev. Biol.* **27**:434.
8. REVEL, J.-P., and K. WOLKEN. 1973. *Exp. Cell Res.* **78**:1.
9. SO, L. L., and I. J. GOLDSTEIN. 1967. *J. Immunol.* **99**:158.
10. EDELMAN, G. M., and C. F. MILLETTE. 1971. *Proc. Natl. Acad. Sci. U. S. A.* **68**:2436.
11. OZANNE, B., and J. SAMBROOK. 1971. *Nat. New Biol.* **232**:156.
12. CLINE, M. J., and D. C. LIVINGSTONE. 1971. *Nat. New Biol.* **232**:152.
13. NOONAN, K. D., and M. M. BURGER. 1973. *J. Biol. Chem.* **248**:4286.
14. TAYLOR, R. B., P. H. DUTTUS, M. C. RAFF, and S. DEPETRIS. 1971. *Nat. New Biol.* **232**:225.
15. YAHARA, I., and G. M. EDELMAN. 1972. *Proc. Natl. Acad. Sci. U. S. A.* **69**:608.
16. INBAR, M., and L. SACHS. 1973. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **32**:124.