# CYCLIC AMP MEDIATES THE CONCANAVALIN A AGGLUTINABILITY OF MOUSE FIBROBLASTS

MARK C. WILLINGHAM and IRA PASTAN

From the Laboratory of Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20014

## ABSTRACT

We have devised a quantitative way to measure the agglutination of cells which utilizes the size discrimination feature of an automatic particle counter. With this method we have studied the agglutinability by concanavalin A of 3T3 cells, a mutant of 3T3 cells (3T3cAMP$^{tcs}$) in which cyclic AMP levels fall when the cells are subjected to temperature change or fresh serum, and L929 cells. We find with 3T3cAMP$^{tcs}$ cells that low levels of cyclic AMP correlate with increased agglutinability and that high levels of cyclic AMP correlate with decreased agglutinability. Prior treatment of these cells with a cyclic AMP phosphodiesterase inhibitor or Bt$_2$cAMP blocks the increase in agglutinability induced by temperature change. When 3T3 cells are treated with fresh serum, their agglutinability also increases although to a much smaller extent than with 3T3cAMP$^{tcs}$ cells. Cells change their agglutinability very rapidly. Treatment of L929 cells for 15 min with 1-methyl-3-isobutyl xanthine at 1 mM decreases their agglutinability to the level of normal 3T3 cells. We conclude that in normal and transformed cells the level of cyclic AMP regulates agglutinability.

A wide variety of studies have now appeared which show that transformed fibroblasts have low levels of cyclic AMP (cAMP) and that treatment of transformed fibroblasts with agents that raise their cAMP levels converts many of their properties toward normal. Those properties of transformed cells affected by cAMP include cell shape (10, 11, 31, 6), growth rate (13, 6, 21, 27, 29), adhesiveness to substratum (14), motility (12), and agglutinability by plant lectins (5, 26, 15). Thus the idea has developed that transforming agents in one way or another lower cAMP levels and that these low cAMP levels are responsible for these changes in cell shape, growth rate, adhesiveness, motility, and agglutinability. To examine this hypothesis further we isolated mutant fibroblasts in which cAMP levels were lowered when the temperature at which the cells were maintained was altered (31). With this mutant cell we have already presented evidence that the fall in cAMP levels is responsible for changes in cell shape and adhesiveness (31). In the current study we have used these cells (3T3cAMP$^{tcs}$-1) to analyze the relationship between cAMP and agglutinability by the plant lectin concanavalin A (con A). We have also studied agglutination of L929 cells and normal 3T3 cells. Our quantitative studies add further evidence to the idea that cAMP regulates the agglutinability of transformed fibroblasts, and also show that cAMP regulates the agglutinability of untransformed cells.

## MATERIALS AND METHODS

### Cell Culture

We obtained and cultured L929, 3T3-4, and 3T3cAMP$^{tcs}$-1 cells as previously described (11, 31). We grew the cells in Dulbecco-Vogt modified Eagle's

medium with 10% calf serum (Colorado Serum Co., Denver, Colo.) and penicillin-streptomycin (50 U/ml each) (Flow Laboratories, Inc., Rockville, Md.) at 37°C in 95% air 5% $CO_2$, and changed the medium every 48 h. $Bt_2cAMP$ and 1-methyl-3-isobutyl xanthine (Searle no. SC2964, G. D. Searle & Co., Skokie, Ill.) (MIX) were prepared as previously described (31).

## Agglutination Procedure

We removed 75-$cm^2$ flasks containing subconfluent cells from the 37°C incubator, poured off the medium, and rinsed twice with 10 ml of phosphate-buffered saline (PBS) ($Ca^{++}$-, $Mg^{++}$-free) at 23°C. After the last rinse, we added 10 ml of PBS with 0.1 mM EDTA to each flask for 5 min at 23°C, and then shook off the cells and spun them in 40-ml conical centrifuge tubes (800 $g$, 5 min). We resuspended the pelleted cells in PBS (without EDTA) and counted an aliquot with a Particle Data Inc. (Elmhurst, Ill.) (model no. 112TA) cell counter. This instrument was used for all the experiments described. 1.5-2.0 × $10^5$ cells were added in 3.0 ml PBS to each 20-$cm^2$ plastic culture dish (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) for the agglutination assay. We solubilized the con A (Miles Laboratories, Inc., Kankakee, Ill.) in saturated NaCl and stored it at 25 mg/ml at 4°C. Fresh dilutions of this stock solution were added to each dish of suspended cells to give final concentrations of 0, 20, 50, and 100 μg/ml. The added volume was less than 0.3 ml per dish.

We found that cells removed by EDTA treatment would settle and stick to both Falconized and untreated plastic dishes in variable amounts whether or not con A was present. Resuspending cells by gentle pipetting after this settling minimized further sticking. Therefore, after the addition of con A to the dishes, each was allowed to sit undisturbed for 3 min at 23°C, and then the contents of each dish were gently pipetted up and down two to three times with a Pasteur pipette. The dishes were placed on a rack in a shaker bath (Fermentation Design, model no. W3325-1) at 23°C and swirled at 25 rpm. After 10 min of incubation, the optimal time for con A-specific agglutination in these experiments, the contents were poured into an Accuvette counting vial containing 17 ml of Isoton counting solution (Coulter Electronics, Inc., Fine Particle Group, Hialeah, Fla.) and assayed as described below. Microscope examination of these dishes after incubation showed cell agglutination comparable to that previously published.

## Spontaneous Agglutination

Spontaneous agglutination or aggregation obscures the measurement of con A-induced agglutination. We observed spontaneous agglutination under two conditions. The first was when we studied 3T3 cells that had become confluent before they were removed from the plastic with EDTA. We found that EDTA readily removed these cells from the dish, but they agglutinated

slightly in the absence of con A during the agglutination assay. Nevertheless, results with confluent 3T3 cells generally agreed with results with nonconfluent cells despite the high background of spontaneous agglutination. The other condition favoring spontaneous agglutination was when the dispersed cells were agitated too long (more than 30 min) or too rapidly (more than 75 rpm) during the agglutination assay.

## Counting Procedure

To quantitate the degree of agglutination in each sample, counts were made with the cell counter lower threshold and current-gain values set to produce a half maximal cell number count, also defined as a standard mean count (SMC), at approximately ¼ of the maximal pulse height (all counts normalized manually for each sample). With the cell lines used in this paper, a current of ½ and a gain of 2⅛ produced a SMC at a lower threshold setting of 150-250 and an upper threshold setting of infinity. This corresponds to a cell volume range of 3,500-6,000 $\mu m^3$ ($K$ = 24.5) in the absence of con A. When cells agglutinated, the height of the pulse produced by clumps passing through the counter orifice was proportionally higher than that produced by a single cell. Theoretically, a two-cell clump with a volume double that of a single cell would have produced a pulse twice the size. If the lower threshold was adjusted above the height of the pulse for a single cell, then most of the counts should be made up of two or more cell clumps. The procedure for quantitation, then, involved counting the number of particles above the lower threshold setting, beginning with 50 and proceeding to 100, 200, 300, and so forth. These counts at each point were then expressed graphically as in Fig. 1 as a percentage of the *total* number of particles in the sample (the number of particles at the *lowest* threshold setting).

## Calculation of Agglutination Index

The counts in the sample without con A were taken to represent the pattern of essentially single cells. From this curve, the SMC and twice the SMC points could be determined (see Fig. 1). For example, the SMC is 240, and twice the SMC is 480. The curve produced by counting in the presence of con A was similarly constructed. By arbitrary choice, the percentage values at 2SMC were taken, and, using these, an artificial agglutination index was constructed as shown in Fig. 1. To compute the agglutination index, the percentage values are read at 2SMC in the presence (A) or absence (B) of the agglutinin:

$$A.I. = \frac{(A) - (B)}{(B)} \times 100.$$

The agglutination index (A.I.) represents the percentage increase (at 2SMC) above the control (without con A) curve due to the presence of con A. The higher the amount of agglutination, the higher the A.I. Duplicate

counts at each threshold point were taken to allow calculation of a range of these values at 2SMC and therefore to give an estimate of the range of the A.I. All experiments reported here were done at least twice, the range representing the average of the ranges of each experiment.

## Con A-Specific Agglutination

When we monitored cellular agglutination in the presence of con A microscopically, two general patterns of agglutination were seen. In one the cell clumps were composed of fewer than 20 cells, mostly two to five cells/clump. In the other the cell clumps were composed of between 20 and 50 cells. In each pattern, the cell clumps were surrounded by a variable number of single, nonagglutinated cells. The reason for these different patterns was unclear, since both patterns were seen in all cell types showing agglutinations, and seem to be independent of the length of time of the agglutination assay or the amount of agglutination seen. Both of these patterns produced an increase in the A.I.

Large cell clumps generally reduced the total number of particles in samples with equal initial numbers of cells. This was evident, then, in lower counts at the lowest threshold point, taken as the total number of particles in the sample. The cell count at 2SMC would not have to represent a very large number of particles to show specific agglutination, since the plotted point was the count expressed as a percentage of the total number of cells in the sample. A similar method of measurement

has already been reported for spontaneous cell aggregation (19). On the other hand, small cell clumps may produce little or no decrease in the total particle number, since apparently the counter may recognize a two-cell clump as two separate cells at the low threshold setting. Small cell clumps produce a marked increase, however, at 2SMC since the pulse height of these clumps is still much higher than that of a single cell. As a result, both large and small cell clumps are detected by this method.

## RESULTS

Fig. 2 shows cells that have been agglutinated with 20 μg/ml con A and have an agglutination index of 53. In assays used by previous workers in which agglutination was expressed on a scale of 0 to + + + +, these cells would be classified as having a low degree of agglutination. Having developed an assay which can quantitatively measure such low degrees of agglutination, we employed the assay to study untransformed 3T3 cells, 3T3-cAMP$^{tcs}$-1 cells in which cAMP levels fall when the cells are placed at a lower temperature, and L929 cells, a commonly used transformed line.

## 3T3-4 Cells

Fig. 3 shows the A.I. values of cells agglutinated with three different concentrations of con A (20,
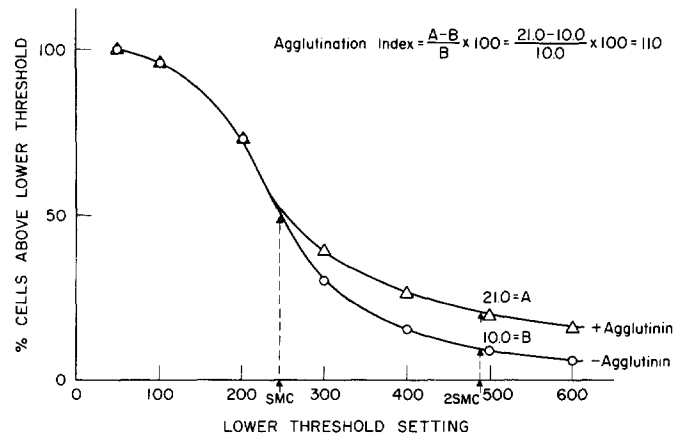


FIGURE 1    Particle size distribution curves in the presence and absence of an agglutinin. This figure shows. the generation of distribution curves produced by counting particle numbers at increasing lower threshold levels. These are then expressed as a percentage of the total number of particles and plotted as shown. Agglutination results in higher counts above the SMC threshold level. The values arbitrarily read at 2SMC in the presence (A) and absence (B) of an agglutinin are used to compute an agglutination index (A.I.) as follows:
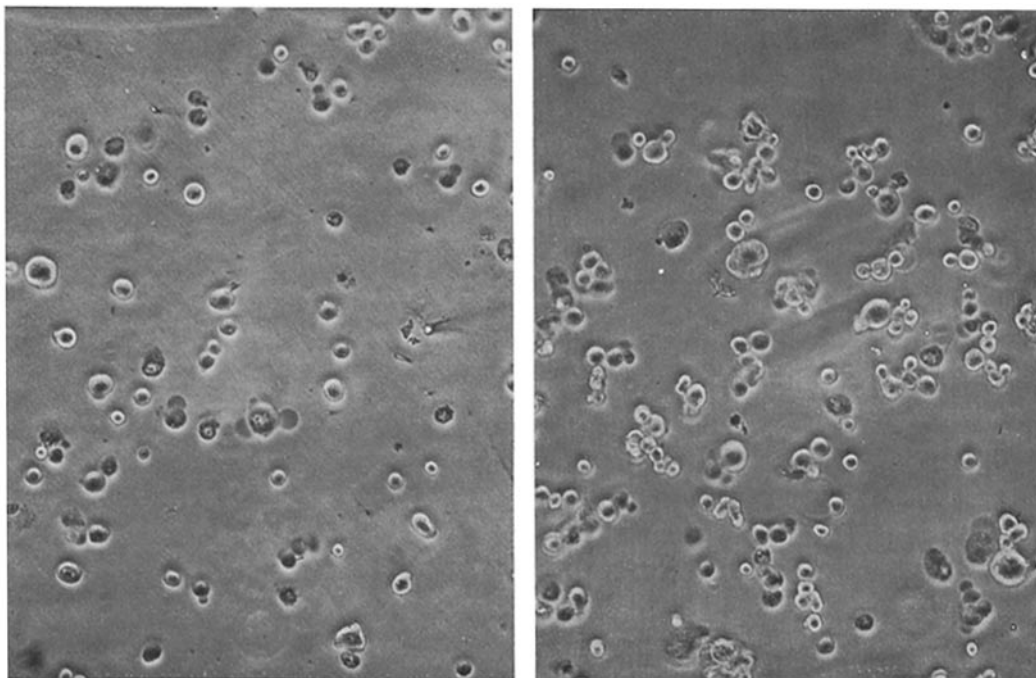
$$A.I. = \frac{(A) - (B)}{(B)} \times 100.$$

FIGURE 2  3T3cAMP^tcs-1 cells agglutinated by Con A after temperature change with new serum addition. These are phase-contrast micrographs of 3T3cAMP^tcs-1 cells after agglutination (10 min). Cells were removed from the incubator and exposed to new 10% calf serum medium for 15 min before removal with EDTA. On the left is a control in the absence of con A, on the right a group of the same cells in the presence of 20 μg/ml Con A. A.I. = 53. × 120.

50, and 100 μg/ml). We observed that normal 3T3-4 cells showed very little con A-specific agglutination either microscopically or by our quantitative method. However, adding new medium with 10% serum to the cells 15 min before removal with EDTA produced a small but significant increase in their agglutinability.

### 3T3cAMP^tcs-1 Cells

When these cells are removed from a 37°C incubator for as little as 30 s, a fall in cAMP levels occurs even if the cells are returned to 37°C. The cAMP levels fall to about 50% of normal after 2–3 min and remain there for about 1 h, then overshoot to very high levels before finally returning to normal 3–4 h later (31). Since it took 20–25 min to measure the agglutinability of the cells, the cAMP levels were low during the course of the agglutination assay. As shown in Fig. 3 and Table I, such cells are much more agglutinable than the parent 3T3 cells. An agglutination assay without temperature change was not possible with the mutant cells, due to their extreme sensitivity to

small changes in temperature. The mutant cells also lower their cAMP levels and show morphologic changes in response to feeding with fresh serum. When temperature shift and feeding were combined, agglutinability with concanavalin A was enhanced (Fig. 3). The increased agglutinability was prevented by a short (15 min) preincubation with 1 mM MIX. This agent was employed because it rapidly increases cAMP levels in many cell types. We also employed Bt$_2$cAMP which acts more slowly in these cells (31). We, therefore, treated cells for 24 h with 1.0 mM Bt$_2$cAMP and found that this concentration of Bt$_2$cAMP also depressed the agglutinability of the 3T3cAMP^tcs-1 cells (Table I).

In the experiments employing MIX, this agent was present during the EDTA treatment, washing, and agglutination assay. If cells pretreated with MIX were prepared and studied in its absence, the effectiveness of the agent was diminished (Table I).

One feature of 3T3cAMP^tcs-1 cells is that 90–120 min after the temperature is lowered, the cAMP levels rebound to levels two to three times
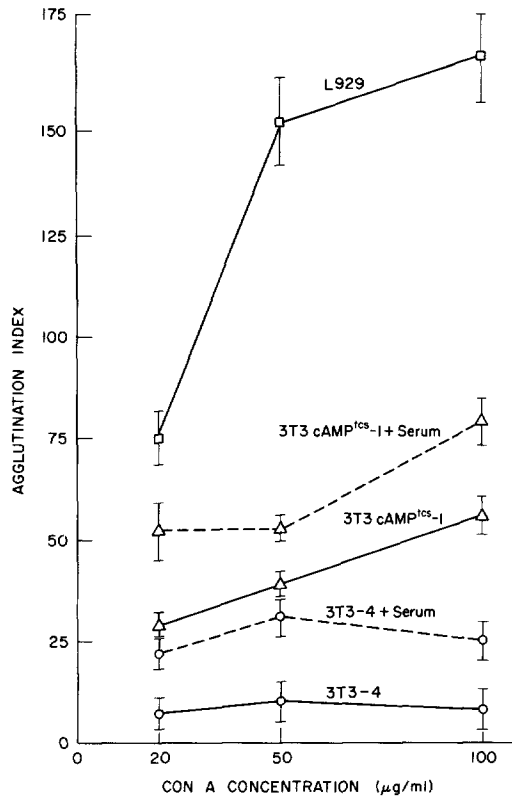
FIGURE 3 Agglutination indices of 3T3-4, 3T3cAMP$^{tcs}$-1, and L929 cells. Each of the cell types were grown and removed with EDTA as described in Materials and Methods. The agglutination indices were calculated from individual experiments with 20, 50, or 100 $\mu$g/ml Con A in the agglutination assay. Cells pretreated with serum were preincubated in a new serum medium for 15 min before removal with EDTA. (O——O) = 3T3-4, (O- -O) = 3T3-4 pretreated with new serum medium, (Δ——Δ) = 3T3cAMP$^{tcs}$-1 cells removed from the 37°C incubator. (Δ- -Δ) = 3T3cAMP$^{tcs}$-1 cells treated with serum medium at the time of temperature change. (□——□) = L929 cells. The bars shown represent the range of the A.I. values at each point.

higher than those of untouched cells (31). We measured agglutinability of cells 90 min after temperature shift and found that their agglutinability was decreased (Table I).

## L929 Cells

As to be expected of transformed cells, L929 cells were highly agglutinable (Fig. 3). After a brief 15-min exposure to MIX or with longer Bt$_2$cAMP treatment, their agglutinability was considerably

reduced (Table I). It is of interest that with both L929 and 3T3cAMP$^{tcs}$-1 cells, washing the cells after the incubation with MIX rapidly increased their agglutinability. This observation may indicate how rapidly cAMP exerts its control over cell agglutinability.

## DISCUSSION

Previously published results with a number of transformed cell lines suggested an intimate relationship between cell surface properties that result in agglutinability with plant lectins, and the transformed state of the cell (8, 7, 1, 4, 3, 9). It became evident after examining many different cell lines that almost all transformed fibroblastic cell culture lines had lower cAMP levels than their corresponding normal parent (21, 27, 20). Further, membrane properties altered in transformation such as adhesiveness (14), morphology (29, 13, 6, 21, 27, 31), and motility (12) seemed integrally related to cAMP metabolism. Therefore, we began to examine whether agglutination with plant lectins was also one of these cAMP-mediated phenomena. Others have already shown that chronic treatment of transformed cells with Bt$_2$cAMP qualitatively decreases their agglutinability (5, 26, 15).

TABLE I

*Agglutination Indices of L929 and 3T3cAMP$^{tcs}$-1 Cells Treated with Agents that Raise cAMP Levels*

| Treatment | (con A = 100 $\mu$g/ml) A.I. | |
| --- | --- | --- |
| | L929 | 3T3cAMP$^{tcs}$-1 |
| Temperature shift | 170 | 56 |
| 1 mM Bt$_2$cAMP (24 h) | 30 | 12 |
| 1 mM MIX (15 min) | 19 | 11 |
| 1 mM MIX 15 min MIX absent during assay | 68 | 29 |
| Recovery phase (90 min after temperature shift) | — | 27 |

L929 and 3T3cAMP$^{tcs}$-1 cells were grown and assayed as described in Materials and Methods. Cells were grown in the presence of 1 mM Bt$_2$cAMP for 24 h or 1 mM MIX for 15 min as shown. For washout of MIX, cells preincubated for 15 min were removed with EDTA, centrifuged, and washed once in 10 ml PBS before the agglutination assay was performed. Recovery phase 3T3cAMP$^{tcs}$-1 cells were obtained by temperature change and serum addition 90 min before removal from the incubator. All assays were in the presence of 100 $\mu$g/ml Con A.

The inability to quantitate agglutination, as well as objections to manipulating cAMP levels by external agents, made this question difficult to answer. The quantitative procedure presented in this paper, and the isolation of an untransformed cell whose cAMP levels could be rapidly lowered (3T3cAMP$^{tes}$-1) (31) made this study practical.

Our findings that the temperature change-sensitive 3T3 cells (a) become agglutinable after temperature shift or after temperature shift with serum addition, (b) show low agglutinability with agents that elevate cAMP levels, and (c) show less agglutination during the refractory period after temperature change, constitute strong evidence that the agglutinability of this cell line is mediated through cAMP. The quantitative confirmation of previous papers (5, 15, 26) that chronic (24 h) Bt$_2$cAMP treatment lowers agglutinability also supports this concept. Our finding that normal 3T3 cells become moderately agglutinable after the addition of new serum-containing medium, a treatment known to lower intracellular cAMP levels in confluent cells (31, 24, 2, 22), indicates that, even in a normal cell, the lower the cAMP level, the more agglutinable the cell becomes. During the cell cycle, cAMP has been found to be low during mitosis, a time when otherwise nonagglutinable cells become highly ag- ˹lutinable 2, 28). One exception, the increased ag-glutinability of Bt$_2$cAMP-treated Chinese hamster ovary cells (30), might be related to direct surface membrane alterations due to the active release of virus induced in these cells by Bt$_2$cAMP.

What is the mechanism through which cAMP regulates agglutinability? Porter et al. (23) have suggested that, since some cell surfaces are smooth during times of nonagglutinability, and studded with microvilli when they are agglutinable, the agglutinability of cells during these times (during mitosis, transformation, or after trypsin treatment) may be related to the presence of these many microvillar structures. Indeed, since the amount of con A bound to cells appears to be the same in cells which do or do not agglutinate (25, 16), then changes in the gross structure of the surface membrane may account for this difference. The points at which agglutinability and microvilli seem to correlate are also points at which cAMP levels in cells are low, suggesting a relationship between cAMP, microvilli, and agglutinability. Nicolson, on the other hand, has suggested that the agglutinability of cells with con

A may be related to the distribution of con A molecules on the cell surface (17, 18).

We feel that the results presented in this paper strongly support the notion that agglutinability is a property to be included in the list of cAMP-mediated phenomena in normal and transformed fibroblasts grown in culture. This in no way implies that this is the only manner by which agglutinability could be regulated. It does, however, point out that in order to show that a transforming agent has caused a specific change in agglutinability, its effects on cAMP metabolism must be considered.

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