AN ANALYSIS OF LECTIN-INITIATED CELL AGGLUTINATION IN A SERIES OF CHO SUBCLONES WHICH RESPOND MORPHOLOGICALLY TO GROWTH IN DIBUTYRYL CYCLIC AMP

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ABSTRACT

We have investigated the molecular basis of the agglutinability of CHO subclones which respond differentially in terms of morphology and surface architecture in the presence of dB-cAMP in the medium. We have demonstrated that the agglutinability of these subclones with both wheat germ agglutinin (WGA) and concanavalin A (Con A) probably depends on the free lateral mobility of the lectin receptor sites in the plane of the membrane. The nonagglutinable surface architecture seems to depend on the presence in the membrane of a protease-labile peptide(s), which appears to be distinct from the lectin receptors, as well as on continuous protein and RNA synthesis. This dependence on continuous transcription and translation may be related to the maintenance of the protease-labile peptide(s) in such a state as to restrict mobility of the lectin receptors. The surface architecture defined as nonagglutinable also depends on the state of polymerization of the intracellular microtubules and microfilaments. It is suggested that these microskeletal elements serve to anchor the lectin receptors in such a manner as to restrict their mobility and thereby reduce the relative agglutinability of a cell line. We suggest that control of the free mobility of both the Con A and WGA receptor sites is dependent on two constraints, one applied by protease-labile ("surface") membrane components and the other by components of the intracellular microskeletal system.

The role of the plasma membrane in controlling cell growth has been an actively investigated area of research over the past few years (7). In particular, many investigators have sought differences in the membrane of normal and transformed cells which might account for the different growth properties exhibited by the two cell types (24). A number of fairly consistent but not invariable types of plasma membrane modification have been reported to occur as a consequence of cell transformation. These have included *(a)* an increase in the average molecular weight of sialofucopeptides released from the transformed cell surface after extensive trypsin digestion (5), and *(b)* enhanced agglutinability of transformed cells by plant lectins (25). It was of some interest, therefore, that the reverse of these changes (i.e. a decrease in the amount of sialic acid associated

with a trypsin-releasable class of fucopeptides [28] and loss of lectin-induced agglutinability [15]) was observed when Chinese hamster ovary cells (CHO)[1] were grown in the presence of dibutyryl cyclic AMP (dB-cAMP).

It has also been demonstrated that CHO cells grown in the presence of dB-cAMP lose their compact, epithelial-like morphology, reverting to a fibroblast-like cell aligned in parallel arrays. Hsie and Puck (14) have described these changes as "reverse transformation."

We have chosen to work with a series of CHO subclones which show a mixed response in terms of the above characteristics after the addition of dB-cAMP to the growth medium. Comparison of the results obtained with these various subclones has permitted an extensive evaluation of lectin-initiated cell agglutination.

MATERIALS AND METHODS

Materials

Cordycepin, cycloheximide, actinomycin D, colchicine, N^6O^2-dibutyryl adenosine 3'5'-cyclic monophosphate (dB-cAMP), and trypsin were purchased from Sigma Chemical Corp., St. Louis, Mo. Cytochalasin B (CCB) was purchased from Gallard-Schleslinger Chemical Manufacturing Corp., Carle Place, N. Y.

Cells

CHO cell line K-1, and subclones M-7 and 24-2 were obtained from Dr. Abraham Hsie, Oak Ridge National Laboratory, Oak Ridge, Tenn. The other line (clone H-7) originated in the laboratory of Dr. R. M. Humphrey, Texas Medical Center, Houston, Tex. All these cells were derived from the original L-proline-requiring CHO clone K-1 of Kao and Puck (17).

The cells were maintained in 75-cm² Falcon flasks (Bioquest, Cockeysville, Md.) in 20 ml McCoy's 5A medium, supplemented with 10% (vol/vol) fetal calf serum, 2 mM glutamine, and an antibiotic-antimycotic mixture. The cultures were maintained at 37°C in a moist incubator in which CO_2 tension was 5% and routinely passaged 1:10 upon reaching approximately 90% confluence.

Purification of Lectins

Wheat germ agglutinin (WGA) and concanavalin A (Con A) were purified from commercially available wheat germ and jack beans, respectively, according to published procedures (19, 1). The purified lectins were lyophilized and stored at −20°C until used.

Determination of Agglutination by Wheat Germ Agglutinin and Concanavalin A

All agglutinations were performed as previously described (23). Care was taken throughout all of the procedures associated with the agglutination assays to maintain the cells in solutions containing the particular concentration of substituted nucleotide or drug which had been applied to the cells in culture.

Cell Synchrony

Cells were grown to confluence in McCoy's 5A + 10% fetal calf serum and then left at the monolayer for 48 h, during which time most of the cells collected in G_1. After this, the cells were replated at lower cell density and allowed to grow for 22 h in McCoy's 5A + 10% fetal calf serum. McCoy's 5A + 0.5% fetal calf serum was then added to the cells for 16 h. This effectively blocked the cells in G_1. After 16 h, medium containing 10% fetal calf serum was returned to the cells, and the cell counts were taken from duplicate plates every 2 h over the next 16 h.

RESULTS

Morphologic Response to dB-cAMP

Hsie and Puck have previously demonstrated that the addition of dB-cAMP to the epithelioid CHO subclone K-1 caused this particular clone to elongate, thereby making it more "fibroblastic" in appearance (14). It was demonstrated at the time that this elongation in the presence of dB-cAMP was dependent on the polymerization of the intracellular microtubules and microfilaments (14, 30).

In order to evaluate what relationship, if any, might exist between the lectin-initiated cell agglutination of individual cells and the morphology assumed by the cells in the presence of dB-cAMP, we have investigated the agglutinability of four independent clones of CHO cells which show varied morphologic response when exposed to dB-cAMP. As can be seen from Table I, only subclones K-1 and H-7 became markedly elongated in the presence of the nucleotide analogue. M-7, whose morphology closely resembled that of K-1 and H-7 under normal growth conditions, showed no visible morphological response to dB-cAMP. Line 24-2, which had a fibroblast-like morphology in the absence of the nucleotide analogue, ap-

[1] *Abbreviations used in this paper:* CCB, cytochalasin B; CHO, Chinese hamster ovary cells; Con A, concanavalin A; dB-cAMP, dibutyryl cyclic 3'5'-adenosine monophosphate; PBS, phosphate-buffered 0.15 M NaCl (pH 7.2); WGA, wheat germ agglutinin.

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TABLE I
Effect of dB-cAMP on the Morphology of the CHO Subclones

Subclone	Morphology	
	Control	+1 mM dB-cAMP
K-1	Epithelioid	Fibroblastic
H-7	Epithelioid	Fibroblastic
M-7	Epithelioid	Epithelioid
24-2	Fibroblastic	Fibroblastic

The determination of the morphology of the subclones relates to the original definitions of Hsie and Puck (14). "Fibroblastic" refers to cells which are extremely thin and elongated, whereas "epithelioid" cells are more compact and do not arrange themselves in longitudinal bundles on the plate surface. For a scanning electron microscope study of the effects of dB-cAMP on CHO cell morphology, see Porter et al. (26).

peared to elongate slightly in the presence of dB-cAMP.

Butyric acid, which might have arisen as a decomposition product of dB-cAMP, had no marked influence on the morphology of any of the cell clones when present in the medium at a concentration of 1 mM.

The morphologic response of K-1 and H-7 could be detected within 2 h of adding dB-cAMP to the growth medium. The cells returned to the epithelioid form 2–4 h after removal of the nucleotide from the medium. As previously reported by Hsie and Puck (14), the change to the fibroblast-like morphology was completely inhibited by the presence of 5×10^{-4} M colchicine in the medium. Similarly, the addition of colchicine to cells grown in the presence of dB-cAMP produced a morphologic reversal to the epithelioid form within 4 h after addition of the drug. The addition of 10 μg/ml cycloheximide or 1 μg/ml actinomycin D to H-7 cells grown in the presence of dB-cAMP did not affect the morphology of the cells even after 4 or 8 h of incubation, respectively. Similarly, the addition of cycloheximide or actinomycin D to H-7 cells immediately before the addition of 1 mM dB-cAMP did not prevent the morphologic transition from the epithelioid to fibroblastic form.

Effect of dB-cAMP on Cell Growth

To determine whether 1 mM dB-cAMP had any significant effect on the growth rate, generation time or final cell density which might have complicated the comparison of the surface structures of cells grown in the presence or absence of the substituted nucleotide, we attempted to evaluate the effect of dB-cAMP on the growth of the subclones.

As can be seen in Fig. 1, the growth rate and final cell density to which the lines grew in the presence or absence of 1 mM dB-cAMP were very similar. The population doubling time of each subclone, in both the presence and the absence of the nucleotide analogue, was approximately 12 h and the final cell density was 2.5×10^5 cells/cm^2. In a few of the experiments, the final cell density of the treated cells did drop off during the last 12 h of investigation. The reason for this drop-off in final cell density is unknown, although it is unlikely to have arisen from a depletion of nutrients since addition of new medium to either the treated or the untreated cells at high cell density did not significantly increase the final cell density of either cell culture. Toxic effects of the nucleotide analogue are unlikely, since cells which have been cultured for many generations in dB-cAMP showed no change in generation time or replating efficiency, even if replated from high cell density.

In order to demonstrate that the growth rate determined for CHO cells grown in the presence of dB-cAMP was not an artefact of a rapidly

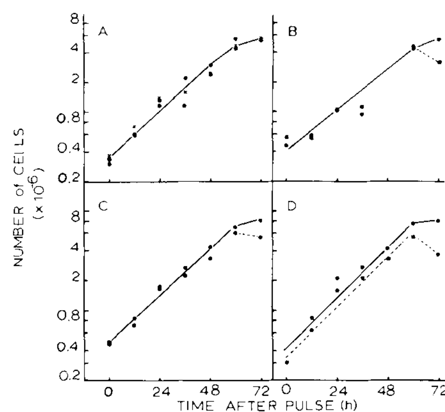

FIGURE 1 Growth of CHO subclones ± dB-cAMP. CHO cells were replated from approximately 80% confluence to 4×10^5 cells per 25 cm^2 flask and allowed to grow in McCoy's 5A medium supplemented with 10% fetal calf serum. Duplicate plates were counted every 12 h. For counting the cells were removed from the plate with 0.25% trypsin and counted in a Coulter counter. The cell counts are expressed as the total number of cells present in a 25-cm^2 tissue culture flask. A, M-7; ○ Control, ● + 1 mM dB-cAMP. B, K-1; ● Control, ○ + 1 mM dB-cAMP. C, 24-2; ● Control, ○ + 1 mM dB-cAMP. D, H-7; ● Control, ○ + 1 mM dB-cAMP.

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cycling subpopulation of cells the H-7 subclone, grown in either the presence or the absence of dB-cAMP, was synchronized. After synchronization in G_1 by serum deprivation, the cells were released from the block and the increase in cell number was monitored. As can be seen in Fig. 2, cells grown in both the presence and the absence of dB-cAMP doubled at the same time, suggesting that the entire cell population maintained its normal doubling time in the presence of dB-cAMP.

Agglutinability of CHO Subclones

We investigated the relative agglutinability of the four CHO subclones primarily to clarify two hypotheses: *(a)* that there may be a correlation between the morphology of a cell and its relative agglutinability (15); and *(b)* that microtubules and/or microfilaments might be involved in modulating cell agglutinability (9, 27).

As can be seen in Table II, all of the subclones, including the elongated 24-2, were readily agglutinated with both Con A and WGA when grown in the absence of dB-cAMP. Table II also demonstrates that an individual cell clone bound approximately equal numbers of lectin molecules when grown in the presence or absence of 1 mM dB-cAMP. It is interesting to note that both the H-7 and 24-2 subclones bound 10^2–10^3 times more WGA molecules per cell when compared to the number of Con A molecules bound.

Table III demonstrates that the relative agglutinability of both the H-7 and 24-2 subclones was dramatically reduced after incubating the cells at

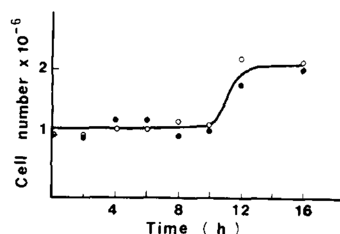

FIGURE 2 Growth of synchronized H-7 cells ± dB-cAMP. H-7 cells were grown as described under "synchronization" in Materials and Methods. Synchronized cells were plated at approximately 1.1×10^6 cells per 25 cm^2 tissue culture flasks. Cell counts were taken of duplicate plates every 2 h after replating. Cell numbers are expressed as total number of cells per 25 cm^2. For counting, the cells were removed from the plate with 0.25% trypsin in PBS and counted in a Coulter counter. ●, H-7 cells grown in McCoy's 5A + 10% fetal calf serum. ○, H-7 cells grown in McCoy's 5A + 10% fetal calf serum + 1 mM dB-cAMP.

0°C in the presence of both Con A and WGA. Reduced agglutinability of the H-7 and 24-2 subclones was also observed after fixation with 0.5% glutaraldehyde (Table IV). It should be noted that prefixation of both the H-7 and 24-2 subclones followed by incubation with 125 μg/ml Con A reduced the agglutinability of both subclones by only 50%. Incubation of the prefixed cells with 12.5 μg/ml Con A, however, produced essentially no agglutination (Table IV), suggesting that at the highest concentration of Con A only limited lectin receptor mobility was necessary to insure agglutination. Prefixation of both cell clones drastically reduced the agglutinability of these clones with 125 μg/ml WGA (Table IV). Extended fixation times (up to 4 h) did not change the data in Table IV.

Taken together, the data relating to incubation of the cell clones at 0°C and glutaraldehyde fixation of the bilayer suggest that Con A- and WGA-mediated agglutination of both subclones was at least partially dependent on the ability of the agglutinin receptors to move laterally in the plane of the membrane. Similar conclusions have been reached by a number of other workers (16, 21, 29).

It has been reported by other workers that incubation of agglutinable cells with vinblastine sulfate to disrupt the intracellular microtubular system rendered such cells nonagglutinable (36). Table III, however, demonstrates that incubating H-7 and 24-2 cells with 0.5 mM vinblastine for 2 h did not reduce the subclones' agglutinability with Con A or WGA. Incubation of either of these subclones with vinblastine for up to 5 h or more similarly had no effect on their agglutinability with either WGA or Con A. That vinblastine is acting intracellularly to disrupt microtubular structures has been strongly suggested by the data of Aubin et al. (2) relating to the need for permeation of the CHO cell by colchicine in order to affect Con A capping.

Incubation of the H-7 or 24-2 clones with 2 μg/ml cytochalasin B (CCB) for 2 h also had no effect on the agglutination of these clones with either lectin, although it did markedly change the morphology of the cells (11), possibly as a result of disrupting the intracellular microfilaments.

Effect of dB-cAMP on Cell Agglutinability

In order to determine the effect of dB-cAMP on the agglutinability of the CHO subclones, each

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TABLE II
Agglutinability of CHO Subclones ± dB-cAMP

Subclones	Agglutinability (%) Con A Control	Con A +1 mM dB-cAMP	WGA Control	WGA +1 mM dB-cAMP	Molecules lectin bound per cell [125I]Con A Control	[125I]Con A +1 mM dB-cAMP	[125I]WGA Control	[125I]WGA +1 mM dB-cAMP
H-7	95	10	80	10	3.1×10^6	6.2×10^6	5.8×10^8	5.7×10^8
24-2	90	20	75	10	1.2×10^6	1.9×10^6	9.2×10^8	1.25×10^9
M-7	100	100	90	100	1.6×10^6	1.4×10^6	NT	NT
K-1	100	100	90	100	NT	NT	NT	NT

All agglutinations and lectin binding were performed as previously described (23). Cells which were grown in dB-cAMP were maintained in dB-cAMP throughout the binding assays. All agglutinations were performed with 125 μg/ml Con A or WGA. Incubation of the lectins with 1 mM concentrations of their respective haptenes before addition of the lectins to the cells reduced agglutinability to less than 10% of the control and lectin binding to 10–15% of the control. The number of lectin molecules bound was determined using a molecular weight of 110,000 for Con A (23) and 34,000 for WGA (19). NT = not tested.

TABLE III
Effect on Agglutinability of Agents Implicated in Modifying Lateral Mobility in the Cell Membrane

Experimental	H-7 −dB-cAMP Con A	H-7 −dB-cAMP WGA	H-7 +dB-cAMP Con A	H-7 +dB-cAMP WGA	24-2 −dB-cAMP Con A	24-2 −dB-cAMP WGA	24-2 +dB-cAMP Con A	24-2 +dB-cAMP WGA
Control	90	85	10	10	95	90	10	10
Incubation at 0°C	10	10	10	10	10	10	10	10
Incubation with 0.5 mM vinblastine sulfate for 2 h	90	80	90	85	70	80	90	80
Incubation with 2 μg/ml cytochalasin B for 2 h	90	90	95	95	90	90	95	95
Incubation with both 0.5 mM vinblastine and 2 μg/ml CCB for 1 h	90	90	95	95	90	90	95	95
Incubation with 10 μg/ml trypsin for 5 min at 22°C	90	90	90	85	90	90	90	85

Agglutinability of Subclones (%)

Cells were grown for 48 h in the presence or absence of 1 mM dB-cAMP before initiation of the described experiments. Preincubation of either lectin with its respective haptene reduced agglutinability to less than 10% of the control. All agglutinations were scored with 125 μg/ml Con A or WGA.

subclone was grown for 48 h in 1 mM dB-cAMP and its relative agglutinability with Con A and WGA was determined. As can be seen in Table II, the presence of dB-cAMP in the growth medium significantly reduced the agglutinability of H-7 and 24-2 with Con A and WGA. The agglutinability of M-7 and K-1 with both lectins was unaffected by the presence of dB-cAMP in the growth medium. It is particularly noteworthy that K-1, which showed a dramatic morphologic response to dB-cAMP (Table I), remained agglutinable with both lectins. The agglutinability of all of the subclones was unaffected by growth in 1 mM butyric acid (data not shown).

Effect of Trypsin

Treatment of the H-7 subclone, which had been grown for 48 h in 1 mM dB-cAMP, with 10 μg/ml trypsin for 5 min at 22°C increased both the Con A- and WGA-initiated agglutinability to that observed in the cell line grown in the absence of dB-cAMP (Table III). This mild protease digestion did not significantly modify the number of lectin receptors available for either Con A or WGA binding (Tables II and V).

Table V demonstrates that the "trypsinized" H-7 subclone, after return to media containing 1 mM dB-cAMP, can "repair" the protease-induced sur-

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TABLE IV

Effect of 0.5% Glutaraldehyde on Con A- and WGA-Mediated Cell Agglutination

Concentration lectin	Percent agglutination							
	H-7				24-2			
	−Glutaraldehyde		+Glutaraldehyde		−Glutaraldehyde		+Glutaraldehyde	
μg/ml	*Con A*	*WGA*	*Con A*	*WGA*	*Con A*	*WGA*	*Con A*	*WGA*
125	90	100	50	50	90	100	50	25
12.5	50	95	10	10	50	95	10	0
1.25	25	50	0	0	25	50	0	0
0.125	0	NT	0	NT	0	NT	0	NT

H-7 and 24-2 subclones were grown for 48 h in McCoy's 5A + 10% fetal calf serum. The cells were then removed from the plate as usual for agglutination and fixed for 1 h at 22°C with 0.5% glutaraldehyde prepared in PBS. After fixation, the cells were washed three times with PBS and then used in the standard agglutination assay. Preincubation of either lectin with the appropriate haptene reduced agglutinability to less than 10%.

TABLE V

Restoration of the Nonagglutinable Surface Architecture after Trypsin Treatment

Time after trypsin treatment (h)	Percent agglutination		Lectin molecules bound per cell	
	Con A	WGA	[^{125}I]Con A	[^{125}I]WGA
0.5	90	90	4.6×10^6	5.0×10^8
1	90	80	4.8×10^6	5.1×10^8
2	50	50	4.8×10^6	5.7×10^8
3	50	20	5.1×10^6	5.3×10^8
4	20	10	6.2×10^6	5.9×10^8

H-7 cells were grown for 48 h in 1 mM dB-cAMP. The cells, still attached to the plate, were washed once with sterile PBS and then incubated for 5 min with 10 μg/ml trypsin at 22°C. After trypsin treatment the cells were again washed with sterile PBS and then medium containing 1 mM dB-cAMP was returned to the plates. Agglutination was scored every hour thereafter with 125 μg/ml Con A or WGA.

face alteration detected as enhanced agglutinability within 4 h after the removal of trypsin. A similar time course for a return to the nonagglutinable state after trypsinization has been reported by Nicolson (20) in 3T3 cells.

Kinetics of the Surface Change

In order to determine the kinetics of the surface change, either from the agglutinable to the nonagglutinable state or from the nonagglutinable to the agglutinable state, cells grown in the presence or absence of the cyclic nucleotide analogue were changed to the opposing medium and agglutination was scored every hour thereafter. Fig. 3 A demonstrates that within 2 h after replacing dB-cAMP-containing medium with medium free of the nucleotide, the agglutinability of clone H-7 with Con A and WGA increased markedly and by 4 h had returned to a state of maximum agglutinability with both lectins. Similarly, the addition of 1 mM dB-cAMP to cells grown in the absence of the nucleotide resulted in a reduced agglutinability within 4 h after addition of the nucleotide (Fig. 3 B).

Effects of Cycloheximide and Actinomycin D on Maintenance of the Surface Architecture

To determine the role of transcription in maintaining the surface architecture detected as lectin-initiated cell agglutination, cells were incubated with 10 μg/ml cycloheximide, 1 μg/ml actinomycin D, or 1 μg/ml cordycepin at the time of the medium change, and the agglutinability with WGA and Con A was followed every hour thereafter. As can be seen in Fig. 4 A, B, addition to the growth medium of 10 μg/ml cycloheximide at the same time as 1 mM dB-cAMP prevented the modification of the surface architecture from the agglutinable to the nonagglutinable state. Fig. 4 C, D demonstrates that the same effect was observed if actinomycin D was added at the time of the medium change.

Addition of cycloheximide, actinomycin D, or cordycepin to the cells immediately after the removal of dB-cAMP from the medium did not prevent the modification of the surface from the nonagglutinable to the agglutinable state (Fig. 5 A, B).

Interestingly, the addition of cycloheximide to cells grown in the presence of 1 mM dB-cAMP without removal of the nucleotide from the growth

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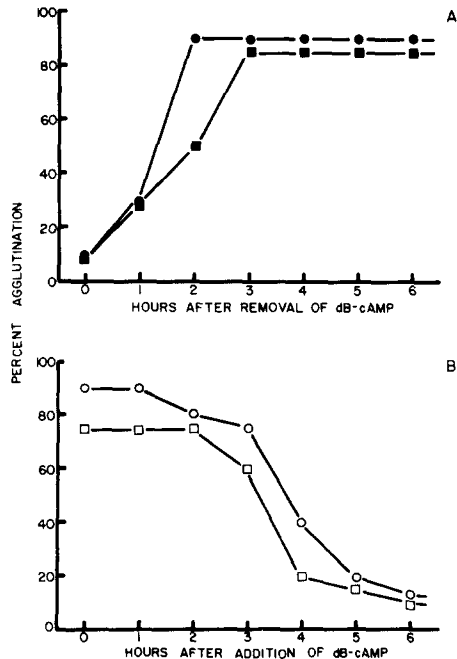

FIGURE 3 Kinetics of the surface change detected as Con A- or WGA-mediated cell agglutination. A. H-7 cells were grown for 48 h in the presence of 1 mM dB-cAMP. At time 0, the cells were washed three times with McCoy's 5A + 10% fetal calf serum and then incubated in this medium lacking dB-cAMP. The relative agglutinability of the cells was then determined every hour with 125 μg/ml Con A and WGA as previously described (11). ●, Agglutinability of H-7 cells with 125 μg/ml Con A. ■, Agglutinability of H-7 cells with 125 μg/ml WGA. B, H-7 cells were grown for 48 h in the absence of dB-cAMP. At time 0, McCoy's 5A medium containing 10% fetal calf serum and 1 mM dB-cAMP was added to the cells and the relative agglutinability of these cells was determined every hour with 125 μg/ml Con A and WGA as previously described (23). ○, Agglutinability of H-7 cells with 125 μg/ml Con A. □, Agglutinability of H-7 cells with 125 μg/ml WGA.

medium resulted in a surface change from the nonagglutinable to the agglutinable state within 4 h after the addition of the drug (Fig. 4 A and B). The time course of the return to the agglutinable state in the presence of cycloheximide was approximately the same as the kinetics observed after the removal of dB-cAMP (Fig. 3 A). The addition of actinomycin D to cells grown in the presence of 1 mM dB-cAMP also resulted in a modification of the surface architecture from the nonagglutinable to the agglutinable state. However, the time course was slower, requiring approximately 6-8 h before the surface returned to the state of maximal agglutinability (Fig. 4 C, D). The addition of cycloheximide or actinomycin D to cells grown in the absence of dB-cAMP did not affect the agglutinability of the cells over the incubation time employed in these studies (Fig. 4 A-D).

Incubation of these cells grown in the presence or absence of dB-cAMP with cycloheximide or actinomycin D for the indicated periods of time did not affect the number of WGA or Con A molecules which can be bound to the cells (data not shown).

Effect of Vinblastine and Cytochalasin B on the Maintenance of the Nonagglutinable State

Fig. 6 A demonstrates that, within 1 h after the addition of 0.5 mM vinblastine sulfate to nonagglutinable H-7 cells, the surface conformation reverted from the nonagglutinable to the agglutinable state. The addition of 0.5 mM colchicine also produced a reversion to the agglutinable state, although in the presence of this drug the reversion was slower, requiring 4 h to produce a complete change to the agglutinable state (data not shown). The addition of 1 mM lumicolchicine (35), a derivative of colchicine which has been demonstrated not to disrupt microtubules, had no effect on the relative agglutinability of the CHO cells (data not shown). Fig. 6 B demonstrates that incubation of nonagglutinable H-7 cells with CCB for 1 h resulted in a dramatic increase in agglutinability of the subclone with both Con A and WGA.

Incubation of H-7 cells grown in the presence of dB-cAMP with both vinblastine and CCB also resulted in an enhanced agglutinability of the H-7 cells (Table III). It must be noted that incubation of cells grown in 1 mM dB-cAMP with colchicine, vinblastine, or CCB for the indicated times also produced a striking change in the morphology of the cells. Both vinblastine and colchicine caused the fibroblastic cells to revert to an "epithelioid" form whereas CCB produced a distinct "rounding" of the cells.

It will, of course, be appreciated that the terms "epithelioid" and "rounded" are simplifications of complicated processes. The scanning electron microscope (EM) work of Everhart and Rubin (11) suggests that the surface morphology of such cells is very complicated (see Discussion).

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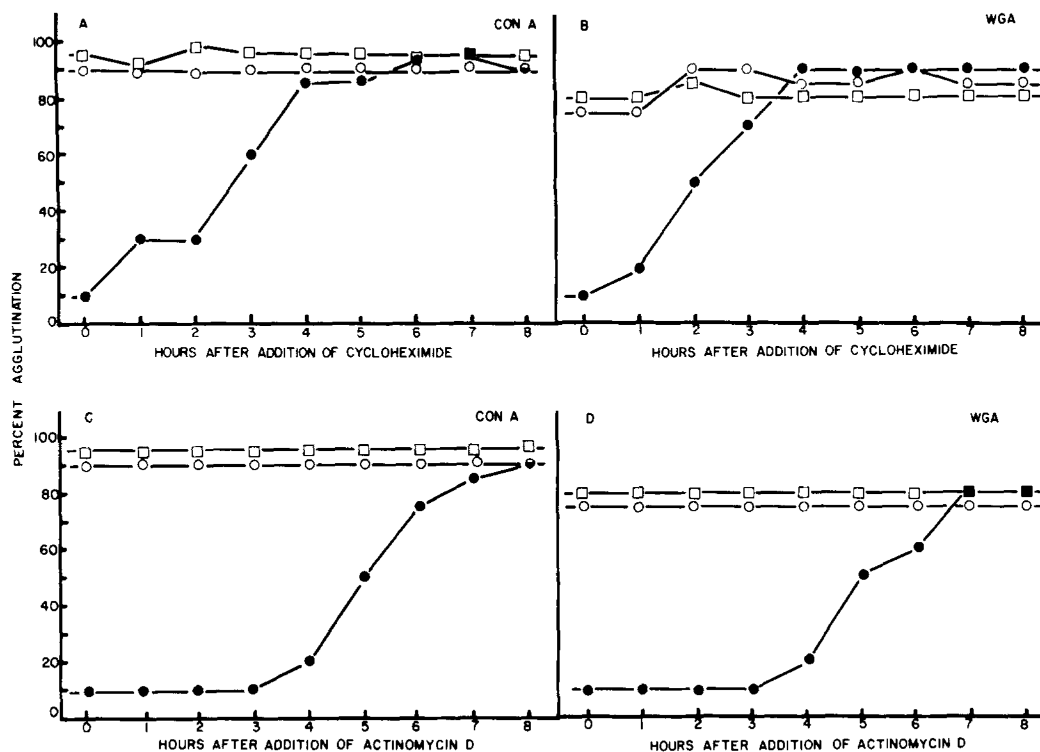

FIGURE 4 Effect of cycloheximide and actinomycin D on the agglutinability of the H-7 subclone grown in the presence or absence of dB-cAMP. H-7 cells were grown for 48 h in either the presence or the absence of dB-cAMP. At time 0 either 10 μg/ml cycloheximide or 1 μg/ml actinomycin D was added to the culture medium and the relative agglutinability of the cell lines was determined with Con A or WGA over the next 8 h. A. Effect of cycloheximide on Con A-initiated cell agglutination. ○, H-7 cells were grown for 48 h in McCoy's 5A + 10% fetal calf serum. At time 0 the medium was changed to McCoy's 5A + 10% fetal calf serum + 1 mM dB-cAMP + 10 μg/ml cycloheximide. Agglutinability with 125 μg/ml Con A was then monitored every hour for the next 8 h. □, H-7 cells were grown for 48 h in McCoy's 5A + 10% fetal calf serum. At time 0, 10 μg/ml cycloheximide were added and agglutinability with 125 μg/ml Con A was monitored every hour for the next 8 h. ●, H-7 cells were grown for 48 h in McCoy's 5A + 10% fetal calf serum + 1 mM dB-cAMP. At time 0, 10 μg/ml cycloheximide were added and agglutinability with 125 μg/ml Con A was monitored every hour for the next 8 h. B. Effect of cycloheximide on WGA-initiated cell agglutination. Same as A, except that agglutinability was monitored with 125 μg/ml WGA. C. Effect of actinomycin D on Con A-initiated cell agglutination. Same as A, except that 1 μg/ml actinomycin D was added rather than cycloheximide. D. Effect of actinomycin D on WGA-initiated cell agglutination. Same as C, except that agglutinability was monitored with 125 μg/ml WGA. It should be noted that these concentrations of cycloheximide and actinomycin D were sufficient to inhibit 90% of the protein and RNA synthesis, respectively, within 1 h after addition of the drug to the medium (data not shown). Essentially the same results in C and D were obtained if the cells were incubated with 1 μg/ml cordycepin in place of actinomycin D.

DISCUSSION

Over the past 5 yr the term "agglutinability" has been used to describe a process in which transformed cells are clumped in the presence of relatively low concentrations of lectin, whereas their normal counterparts remain as single cells. This process was originally conceived as resulting from a simple cross-linking of cells by the multivalent lectins. However, many laboratories have demonstrated that agglutinability with at least one plant lectin, Con A, involves multiple facets of cell surface chemistry (22). Among the various parameters which have been implicated in maintaining the

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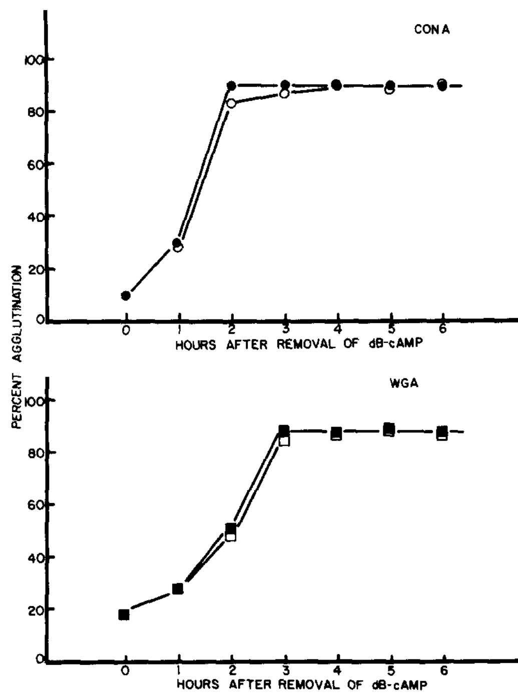

FIGURE 5 Effect of cycloheximide and actinomycin D on the change of surface architecture from the nonagglutinable to the agglutinable state. A. H-7 cells were grown for 48 h in McCoy's 5A + 10% fetal calf serum + 1 mM dB-cAMP. The cells were then washed three times with McCoy's 5A + 10% fetal calf serum and finally incubated in McCoy's 5A + 10% fetal calf serum + 10 μg/ml cycloheximide (●) or 1 μg/ml actinomycin D (○). Agglutination was scored with 125 μg/ml Con A. B. Same as A, except that agglutination was scored with 125 μg/ml WGA. ■, 10 μg/ml cycloheximide added at time of medium change. □, 1 μg/ml actinomycin D added at time of medium change.

surface structure defined as enhanced agglutinability with Con A are the number of lectin-binding sites (23), the mobility of lectin receptors in the plane of the membrane (16, 21, 29), the microskeleton (32, 27, 36) and protease-labile surface structures (6, 20), and others (22).

One difficulty which has been encountered in further characterizing the molecular basis of cell agglutination has been the lack of a cell line in which the modulation of the surface conformation from the agglutinable to the nonagglutinable state is rapid enough to allow one to follow the surface change of a "synchronous" population. The CHO cell clones discussed here were ideal for such a study since the transition from the agglutinable to the nonagglutinable state occurred within 4 h after the addition or removal of dB-cAMP. Our evidence suggests that a number of cellular components are involved in determining both the Con A- and WGA-mediated agglutinability of the investigated CHO subclones.

Lectin Receptors

All of the CHO clones tested showed extensive binding of both Con A and WGA to specific receptors on the cell surface. Our evidence, like that of many other investigators (22), demonstrates that the relative agglutinability of each cell clone was not determined by the number of lectin receptors available on the cell surface. This is

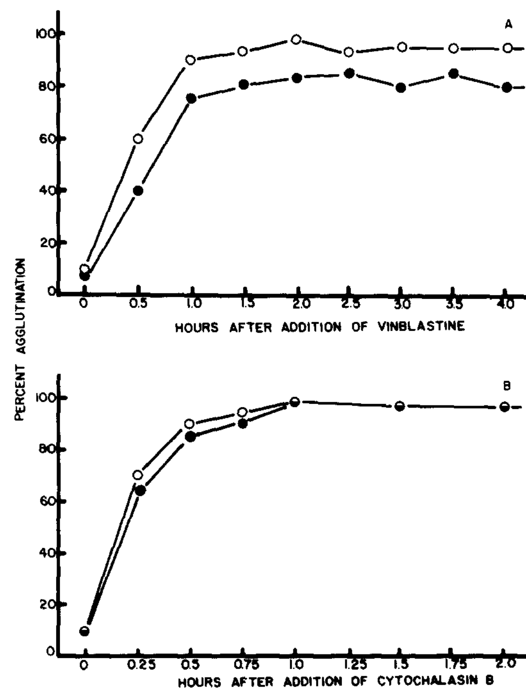

FIGURE 6 Kinetics of the effect of vinblastine sulfate and cytochalasin B on Con A- and WGA-initiated cell agglutination. A. H-7 cells were grown for 48 h on McCoy's 5A + 10% fetal calf serum and 1 mM dB-cAMP. At time 0, 0.5 mM vinblastine sulfate was added to the culture medium and agglutinability with 125 μg/ml Con A and WGA was determined every 0.5 h thereafter. ○, Agglutinability with 125 μg/ml Con A. ●, Agglutinability with 125 μg/ml WGA. B. H-7 cells were grown for 48 h on McCoy's 5A + 10% fetal calf serum and 1 mM dB-cAMP. At time zero, 2 μg/ml cytochalasin B were added to the culture media and agglutinability with 125 μg/ml Con A and WGA was determined every 0.25 h thereafter. ○, Agglutinability with 125 μg/ml Con A. ●, Agglutinability with 125 μg/ml WGA.

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strikingly demonstrated in the H-7 subclone which binds approximately two times more Con A when grown in the presence of dB-cAMP compared to the amount bound in the absence of dB-cAMP, but which shows little agglutinability with Con A when grown in media containing dB-cAMP. This finding is in distinct opposition to our previous findings in 3T3 cells (23) and suggests clearly that each cell line must be treated independently. The data reported in this manuscript suggest that increased lectin-binding is neither necessary for nor sufficient to explain the enhanced agglutinability of some cell lines.

Mobility of Lectin Receptors

As has been demonstrated with a variety of other cell lines (16, 21, 29), incubation of the CHO subclones at 0°C or prefixation with glutaraldehyde blocked the agglutinability of the various subclones grown in the absence of dB-cAMP. In agreement with the work of others, this suggests that the free mobility of the lectin receptors in the plane of the membrane is an essential facet of the agglutination process.

Role of Surface Proteins in Modifying Agglutinability

Treatment of the nonagglutinable CHO clones with low concentrations of trypsin rendered the cells as agglutinable as cells grown in the absence of dB-cAMP. This suggests that protease-labile surface structures probably play a role in controlling the relative mobility of the lectin receptors and, subsequently, the relative agglutinability of the cells with Con A and WGA.

It must be noted, however, that an alternative interpretation of the protease data is possible. Willingham and Pastan (33) have suggested that cell agglutination is dependent on the levels of intracellular cAMP which, in turn, determines the topologic distribution of microvilli on the cell surface (34). Follett and Goldman (12) have demonstrated that trypsinization of BHK cells significantly increases the number of surface microvilli. Thus, trypsinization of the nonagglutinable CHO cells could enhance agglutinability by increasing the number of surface microvilli. Such an interpretation is presently being investigated in our laboratory.

Rapidity of the Surface Change

The H-7 subclone modified its surface conformation from the agglutinable to the nonagglutinable state within 4 h after the addition of dB-cAMP to the medium. This modification was dependent on both RNA and protein synthesis. Return to the agglutinable state after the removal of dB-cAMP also required 2–4 h. However, this surface modification was independent of protein or RNA synthesis.

Maintenance of the Nonagglutinable State

Maintenance of the nonagglutinable state was dependent on continuous protein and RNA synthesis. From this evidence, we would suggest that maintenance of the nonagglutinable surface architecture on these CHO subclones is dependent on the presence of protease-labile surface peptides whose association with the lectin receptors is dependent on continuous transcription and translation. Similar conclusions have been reached by Baker and Humphreys (3) as well as Borek et al. (4) in work performed with other cell lines.

Our work with vinblastine and cytochalasin B suggests a role for both the microtubule and microfilament systems in maintaining the nonagglutinable surface architecture. Such a role has been suggested by a number of other workers (32, 9, 27).

In our opinion, there are a number of alternate explanations for the effect of vinblastine and cytochalasin B on the maintenance of the nonagglutinable state of the CHO cells. One possibility is that the intact microtubules and microfilaments behave as stabilizing agents directly responsible for limiting the mobility of surface structures, as proposed in references 9, 22, 27, and 36. Such a function could result from a direct association of the microtubules or microfilaments with the lectin receptors or might result from the formation of a meshwork on the inner side of the plasma membrane in a manner analogous to that recently proposed for "spectrin" (10). Electron microscope observation does suggest that microfilaments may be found in association with the plasma membrane (13). However, there is little evidence to support a direct association of microtubules with the plasma membrane (31). A second possibility which must be considered as an explanation for the effect of vinblastine and cytochalasin B on maintenance of the nonagglutinable surface architecture is that the microtubules and microfilaments serve to control the presence and distribution of such surface structures as microvilli. Work by Willingham and Pas-

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tan (33, 34) suggests that such macrostructures may play an essential role in determining the relative agglutinability of a cell line. A third possibility which must at least be considered is that the effects of vinblastine or cytochalasin B that are unrelated to their effects on the microskeletal systems are responsible for the observed results. That vinblastine is acting intracellularly on CHO microtubules has recently been strongly suggested by Aubin et al. (2), and Ukena et al. (32). A direct demonstration that the effect of cytochalasin B on agglutinability is directly traceable to its effect on microfilaments is more difficult. Goldman (13) has demonstrated that CCB does not act to dissociate all of the intracellular microfilaments in BHK21 cells. Kletzien and Perdue (18) as well as Cohn et al. (8) have demonstrated that CCB acts to inhibit sugar uptake, presumably by binding to the sugar transport site. As a result of scanning EM studies, Rubin and Weiss (30) have suggested that CCB acts at the cell surface to modify surface macrostructures.

It is of interest to note that incubation of agglutinable CHO cells with colchicine does not affect the relative agglutinability of the cells as it does in the SV3T3 system (32). This work implies that dissociation of the microtubules does not result in the capping of the Con A receptors. This result has recently been supported by the work of Aubin et al. (2), and our own unpublished work dealing with the patching and capping of fluorescently labeled lectins. Only a coordinated biochemical and morphologic evaluation of a cell system such as is offered by these CHO cell clones will allow us to distinguish between these three alternative interpretations.

Despite the difficulties inherent in using the various drugs alluded to in the above paragraphs, it is clear from our work with these CHO subclones that simple polymerization of the microtubules is not in itself sufficient to reduce the agglutinability of a subclone. This is shown most graphically by the K-1 subclone which, although it responds morphologically to dB-cAMP, nevertheless remains agglutinable with both Con A and WGA. As we have mentioned, this morphologic response is dependent on the polymerization of the microtubules. The fact that the cells remain agglutinable in the presence of dB-cAMP suggests either that a class of microtubules separate from those that determine morphology is responsible for reducing the mobility of surface structures, or that a secondary interaction of the lectin receptors with the polymerized microtubules is essential for reducing the mobility of the lectin receptors.

Thus, in the CHO cell clones described, we would suggest that the accumulated data implicate protease-labile surface structures as well as the cellular microskeletal elements as the cellular components capable of stabilizing the relative mobility of the lectin receptors and thereby determining the relative agglutinability of a cell clone. We would suggest that limited proteolysis or disruption of the microskeletal system can release the restraint to lectin receptor mobility and thus permit agglutination to occur.

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