# AGGLUTINATION OF A TRANSFORMED MOUSE CELL LINE AND A VARIANT SUBLINE WITH CONCANAVALIN A: EFFECT OF TEMPERATURE AND TIME OF LECTIN INCUBATION

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SUMMARY. Colchicine treatment enhanced Con A-mediated agglutination of erythrocytes to LM cells (LM is a "spontaneously" transformed mouse line) incubated for brief periods with Con A at 22° C. Longer incubations with Con A at 22° C rendered colchicine treated cells less agglutinable than untreated cells. Even short incubation times with Con A at higher temperature (37° C) rendered colchicine treated LM cells less agglutinable than their untreated counterparts. Below 15° C, colchicine treated cells remained more agglutinable than untreated cells even after long periods of Con A treatment. Cells of a variant clone (R1) isolated from LM by negative selection with concanavalin A exhibited increased substratum adhesiveness and an absolute serum requirement. LM and variant cells exhibited a differential reponse to colchicine treatment, the variant subline reguiring longer periods of colchicine treatment to elicit changes in morphology and agglutinability.

The differentially enhanced concanavalin A (Con A)-mediated agglutinability of certain transformed vs normal cell lines may be a consequence of increased Con A receptor mobility (1-3). Other surface receptors, e.g., H-2 antigen, also appear to have increased mobility following transformation of 3T3 cells (4). Differences in membrane lipid fluidity are unlikely to account for this behavior (5,6). Nicolson (1,6), Berlin (7), and Edelman (8) have proposed that lateral mobility of certain surface components may be restricted by their association with submembranous cytoskeletal structures. Colchicine and other microtubule-disrupting agents can alter the inherent organization of some cell surface macromolecules including Con A receptors (9). In addition, Berlin's group has shown that these drugs dramatically inhibit the Con A-mediated agglutination of erythrocytes to SV3T3 cells. Using a spontaneously transformed mouse fibroblast line (LM), we observed an enhancement of Con A-mediated hemadsorption after treatment of LM cells with



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colchicine or vinblastine (11). We now present evidence which may reconcile these contradictory findings.

MATERIALS AND METHODS. LM cells derived from NCTC clone 929 (L cells) were maintained in Eagle's minimal essential medium (MEM) with Hank's salts plus 0.5% bactopeptone (MEM + P)(12). The cells were free of mycoplasma as determined by the method of scanning emission electron microscopy (13). We are grateful to Dr. J.P. Revel (California Institute of Technology) for these analyses.

Variants resistant to the cytotoxic action of Con A were isolated by the method of Ozanne (14). The flat variant clone (R1) described in this study was used between the fifth and fifteenth passages after cloning and maintained on MEM + 10% calf serum (GIBCO).

For studies of colchicine effects on the Con A-mediated agglutination of rabbit erythrocytes to cells (hemadsorption), cultures were used at 80-100% confluency. Colchicine (Sigma) was dissolved in 0.85% (w/v) NaCl immediately before use and added at 5 x $10^{-6}$ M to cells in culture medium (MEM).

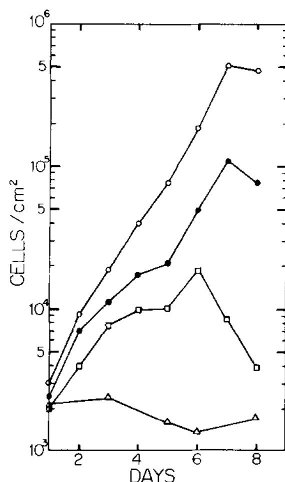


FIGURE 1. Saturation densities of R1 and LM cells. Cells of the transformed line, LM, and of a Con A resistant variant subline, R1, were grown in multiwell dishes (35 mm diameter) and duplicate independent trypsinized suspensions were counted in a hemacytometer for determination of cell number. LM cells grown in MEM + 0.5% bactopeptone (○—○); R1 cells grown in MEM + 5% calf serum (●—●); R1 cells grown in MEM + 1% calf serum (□—□); and R1 cells grown in MEM + 0.5% bactopeptone (△—△).

RESULTS. The Con A-resistant variant (R1) exhibited a number of phenotypic properties usually associated with untransformed cells. The variant cells have an absolute serum requirement, whereas LM cells grow in MEM + 0.5% bactopeptone (Figure 1). The adherence of R1 cells to substratum was greater than that observed with LM cells (Figure 2).

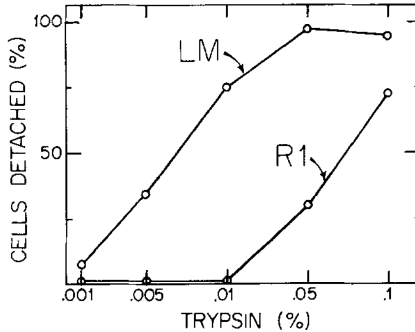


FIGURE 2. Adhesion of LM and R1 cells to the substratum. Cells of both the transformed line (LM) (grown in MEM + 0.5% peptone) and the variant subline (R1) (grown in MEM + 10% calf serum) were grown to a 90-95% confluent monolayer in multi-well dishes (33 mm diameter). The cells were treated with trypsin at the indicated concentrations for 10 min at 37° C and the dishes were gently rotated for 1 min. The cells that detached were then counted in a hemacytometer. The cells that remained attached were removed by incubation with 0.25% trypsin for 15 min at 37° C and were also counted. The percentage of cells detached from the substratum is the number of cells removed by the initial trypsin treatment divided by the total number of cells originally present in the well. All points are the average of six determinations. Note the change in scale on the abscissa of 0.1% trypsin.

Con A-mediated agglutinability was assayed by a hemadsorption technique which measures the adsorption of red blood cells to fibroblasts attached to the culture dish (10,15,16). The variant subline was less susceptible to Con A-mediated hemadsorption than was the parental transformed line (Figure 3). R1 cells required over 5-fold more Con A to achieve the level of hemadsorption mediated by LM cells incubated with 100 μg/ml of Con A. This reduction in lectin-mediated agglutinability is apparently not the result of a smaller number of Con A binding sites since both cell lines contain approximately $2 \times 10^7$ per cell (Table 1).

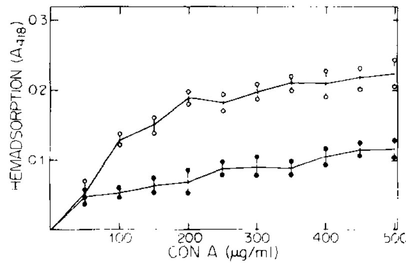


FIGURE 3. Con A-mediated hemadsorption to LM and R1 cell monolayers. Cells were grown in MEM + 15% salt fractionated serum (23) in Linbro multi-well dishes (35 mm diameter) to an 85-95% confluent monolayer. The cells were washed twice with saline (0.85% NaCl) and incubated for 5 min at 22° C with the indicated concentrations of Con A in PBS (Table 1). The cells were then washed five times with saline and incubated for 10 min at 22° C with rabbit erythrocytes (1% v/v) in PBS. Finally, the cells were washed five times with saline, solubilized in 5% sodium dodecyl sulfate (w/v) and analyzed spectrophotometrically for hemoglobin content at 418 nm. Transformed (LM) cells (○—○); and Con A-resistant variant (R1) cells (●—●).

TABLE 1. Binding of Concanavalin A to R1 and LM Cells.

| Concentration of labeled Con A (μg/ml) | Con A bound (cpm/mg of cellular proteins) | |
|---|---|---|
| | R1 cells | LM cells |
| 50 | 31,000 ± 1,600 | 31,700 ± 1,700 |
| 100 | 43,400 ± 2,200 | 44,440 ± 2,700 |
| 200 | 50,200 ± 1,700 | 48,500 ± 1,800 |

Cells were grown in MEM + 15% salt fractionated serum (23) in multi-dish wells (35 mm diameter) to an 85-95% confluent monolayer. The medium was aspirated from the wells, and the cells were washed twice with saline (0.85% NaCl) and incubated for 5 min at 22° C in PBS solution (0.1 M sodium phosphate buffer at pH 7.2, containing 0.85% NaCl and 1 mM $MgCl_2$) containing $^3H$-labeled Con A at the indicated concentrations. Following this incubation, the monolayers were washed five times with saline at 22° C, and cellular material was solubilized and assayed for radioactivity as described previously (16). The specific activity of $^3H$-acetate labeled Con A (21) was 5.7 x $10^6$ cpm/mg of protein, determined by the procedure of Lowry _et al_. (22). Each value represents the average of six determinations, and values are expressed ± the standard deviation.

Figure 4 describes the effects of temperature and colchicine on Con A-mediated agglutination of LM cells. In these experiments, cell monolayers

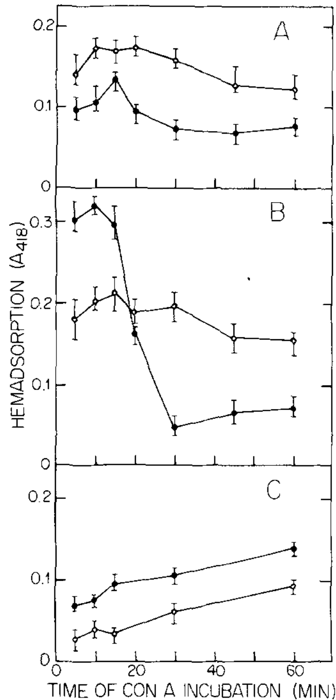


FIGURE 4. Effect of temperature and time of incubation with Con A on Con A-mediated hemadsorption to colchicine-treated and untreated LM cells. LM cells were grown in MEM + 0.5% bactopeptone in multi-well dishes (35 mm diameter) to an 85-95% confluent monolayer. The hemadsorption assays were performed with 100 µg/ml of Con A exactly as described in the legend to Figure 3, except for the temperatures and time periods used in the incubations. Cells were incubated for 3 hours at 37° C in the presence or absence of 5 x $10^{-6}$ M colchicine (Materials and Methods). Treatment with colchicine for this period of time brought about a rounded appearance in cell shape as observed by phase contrast microscopy. Cells incubated with colchicine for 3 hours prior to hemadsorption assay (●—●). Cells incubated in MEM in the absence of colchicine for 3 hours prior to hemadsorption assay (O—O). In A, the incubations with Con A and with rabbit erythrocytes for hemadsorption were at 37° C. In B and C, these incubations were performed at 22° C and 10° C, respectively.

were incubated with or without colchicine until significant cell morphology changes were observed when colchicine was present. Both the treated and untreated cells were then exposed to Con A at 37° C, 22° C, and 10° C for various periods of time, and agglutinability was determined by the hemadsorption assay. Incubation with Con A at 37° C for even short periods of time led to reduced agglutinability of the colchicine treated vs. untreated cells

(Figure 4A). This is essentially the observation previously reported by Berlin and his associates for SV3T3 cells (10,17) and is just the opposite of that expected if increased cell surface mobility results from cytoskeletal breakdown. However, excessive receptor redistribution could lead to reduced agglutinability. If this is the case, a lower temperature of incubation with Con A might alter the effects of colchicine observed at 37° C.

The experiment in Figure 4B indicates that colchicine treated cells were more agglutinable than untreated cells following a short incubation period with Con A at 22° C. Prolonged incubation of colchicine treated cells with Con A at 22° C led to a loss in agglutinability, rendering them less agglutinable than the untreated cells. When the incubation with Con A was conducted at 10° C (Figure 4C), colchicine treated cells were more agglutinable than untreated cells for extended incubation periods with Con A.

Cells of the Con A-resistant variant were not similarly affected by colchicine under conditions used with LM cells. Extended incubation of R1 cells with colchicine did result in a slight rounding of cellular shape. An increase in Con A-mediated hemadsorption was also oberved for R1 cells subjected to this treatment (Figure 5).

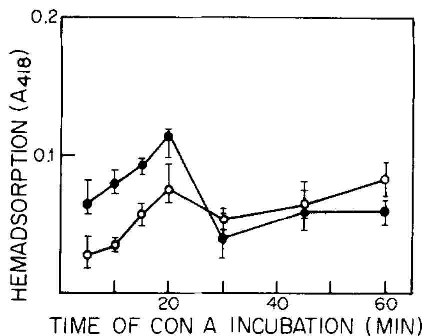


FIGURE 5. Effect of colchicine on Con A-mediated hemadsorption to R1 cells. The variant (R1) cells were grown in MEM + 10% calf serum in 35 mm diameter multi-well dishes to 85-95% confluent monolayers. The hemadsorption assays were performed at 22° C as described in the legend to Figure 3, except for the time period of incubation with 100 µg/ml of Con A. Cells incubated in the presence of 5 x $10^{-6}$ M colchicine at 37° C for 6 hours before assaying hemadsorption (●—●). Cells incubated for 6 hours at 37° C in the absence of colchicine (○—○).

DISCUSSION. The studies reported here indicate that cytoskeletal integrity is one property affecting agglutination. Colchicine treatment was reported to markedly decrease the Con A-mediated agglutinability of transformed 3T3 cells (10), a result in direct contradiction with that expected. The studies reported in Figure 4 indicate that colchicine treatment can render transformed cells more agglutinable under defined conditions but that incubation of colchicine-treated cells with Con A for excessive periods of time or at a higher temperature can lead to reduced agglutinability. Incubation of colchicine-treated LM cells with Con A results in capping of Con A receptors, using the hemocyanin technique to assess receptor distribution (P. Linsley and C.F. Fox, unpublished observations). Redistribution of Con A receptors, leading to capping and perhaps even concomitant pinocytosis of regions containing patched or capped receptors, could reduce the availability of receptor sites for agglutination. Berlin and his associates have observed that colchicine treatment of transformed 3T3 cells results in a cap-like aggregation of Con A binding site clusters upon lectin treatment and concluded that this might explain the reduced Con A-mediated agglutinability which they observed with colchicine-treated, transformed 3T3 cells (17). Sach's group has used fluorescent and ferritin-labeling techniques to demonstrate that Con A-mediated agglutination of fibroblasts and lymphocytes may require a final clustered distribution of Con A binding sites and is severely inhibited by cap formation of Con A binding sites (18).

Colchicine prevents the polymerization of tubulin to form microtubules (19) and apparently affects surface topography and agglutinability as a consequence of its disruptive action on microtubule structure (10,11). Cytoplasmic structures localized on the intracellular surface of the plasma membrane might affect lectin-mediated agglutinability by directly anchoring proteins which span the membrane, thereby restricting their lateral mobility. These cytoplasmic substructures could include microtubules or structures which might react with microtubules, e.g., microfilaments. The variant cell

subline (R1) was more resistant to the action of colchicine than were the parent LM cells (Figures 3 and 4). One explanation for the increased resistance of untransformed cells to colchicine action is a more rigid cytoskeleton endowed by augmentation of cytoskeletal structures. In this regard, a more extensive microfilament network has been observed in 3T3 cells compared to viral transformed 3T3 cells (20).

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