

CORRELATION BETWEEN MOVEMENT OF CONCANAVALIN A MEMBRANE RECEPTORS AND CYTOLYSIS

A Scanning Electron Microscopy Study

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ABSTRACT

The present study was undertaken to test whether cytolysis induced by Concanavalin A (Con A) requires lateral mobility of membranal lectin receptor sites into caps. Treatment of interphase murine mastocytoma cells with 10^{-4} M colchicine promoted cap formation by Con A in about 30% of the cells, followed by cytolysis. Pretreatment of the cells with NaN_3 , low temperature, or glutaraldehyde decreased the degree of capping and, to the same extent, the degree of cytolysis. The addition of antibodies to cells bound with Con A increased the appearance of capping and cytolysis. A linear relationship with a high correlation coefficient exists between the degree of capping and cytolysis, suggesting that lateral mobility of membrane Con A receptors is required for cytolysis by the lectin. The process of cap formation by Con A up to the stage of cytolysis was followed by scanning electron microscopy.

KEY WORDS Concanavalin A · membrane receptors · cytolysis · scanning electron microscopy · capping

As a consequence of the specific binding of lectins to receptors on the cell membrane, various changes occur in the cell and on the membrane itself. One effect of the binding of multivalent lectins is the redistribution of the lectin-bound receptors on the cell surface, leading to the phenomena of clustering and capping. Another consequence of the binding of some lectins is the induction of DNA, RNA, and protein synthesis. Furthermore, most lectins have been found to have a cytotoxic effect, although the exact mechanism leading to cytolysis is, in most cases, unknown (for review, see Nicolson [20]). In attempting to understand this mechanism, we have recently shown

that various lectins cause a similar cytolysis in normal murine bone marrow and mastocytoma cells (14). In addition, it would be of interest to know whether the two phenomena of lectin-induced capping and lectin-induced lysis are related. Using a membrane mobility agent with wheat germ agglutinin (WGA), our group has recently presented evidence supporting the hypothesis that capping leads to lysis (16). In a later study (13), we have reported that preincubation of interphase murine mastocytoma cells with trypsin-promoted cap formation by WGA was followed by cytolysis of these cells. It was suggested that lateral mobility of membrane WGA receptors is required for cytolysis produced by the lectin. Further studies have shown that restricted small-hole formations followed by osmotic lysis are responsible for the damage induced by WGA in trypsin-treated mas-

toctoma cells. However, incubating such cells in nonpenetrating solutes such as dextrans of high molecular weight could protect them from lysis.

To test experimentally the hypothesis that cap formation by multivalent lectins is required in the cytolytic effect, it is desirable to have an experimental system of cells in which capping can be induced, inhibited, and enhanced. It is known that Concanavalin A (Con A) can induce formation of caps in some types of cells (3, 6, 11, 18, 23) and that cap formation can be increased in normal lymphocytes or fibroblasts by treatment with colchicine (3, 6, 15, 27, 34) which binds to tubulin (17, 30, 31, 32).

The present study demonstrates that Con A induces caps followed by lysis in colchicine-treated mastocytoma cells. By using scanning electron microscopy (SEM), all stages of the lytic process were demonstrated.

MATERIALS AND METHODS

Cells

A mastocytoma cell line (P-815-X2) (5) was used in all experiments. The cells were grown in Dulbecco's modified Eagle's medium (GIBCO, Grand Island Biological Co., Grand Island, N.Y.) supplemented with 10% heat-inactivated horse serum. The cell line was maintained by transferring 10^5 cells to 5 ml of fresh medium every 4-5 days. Cell populations with low mitotic indices (less than 1%) were used in all the experiments ("interphase cells"). The cells were washed and suspended in phosphate-buffered saline (PBS) containing 140 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4 , 1.5 mM KH_2PO_4 at pH 7.3.

Lectin

Con A unlabeled and labeled with rhodamine (Rh-Con A) and goat anti-Con A antibodies (1 mg antibodies/ml) were obtained from Yeda Research and Development Co., Ltd., (Rehovoth, Israel). Succinyl-Con A was prepared in our laboratory (15).

Cytotoxicity Test

Cytotoxicity was determined by counting the cells in a 0.4% trypan blue solution in PBS. In samples treated with Con A, cell counting was performed after disaggregation of cell agglutinates to single cells by the addition of 0.1 M α -methyl-D-glucoside; the hapten inhibitor sugar. The percent of lysis was determined by comparing the number of whole cells found after incubation with Con A to the number of whole cells found after incubation without the lectin, but treated with the hapten inhibitor sugar.

Fluorescence Microscopy

The distribution of Rh-Con A on the cell surface was observed with a Zeiss incident light fluorescence microscope. The same slides were also examined with a phase-contrast microscope.

Scanning Electron Microscopy (SEM)

For SEM, the cells were incubated with Con A and then fixed in 1% glutaraldehyde in PBS for 60 min, rinsed twice with PBS and then allowed to sediment (no centrifugation) for 24 h on cover slips coated with poly-L-lysine. The cells were dehydrated in a graded series of alcohols followed by Freon 113 for 5 min each. The specimen was then quickly transferred to a previously cooled high-pressure chamber of a critical-point drying apparatus (Polaron, England) and critical-point dried in carbon dioxide. The cells were coated with gold and examined with a Philips 500 PSEM scanning electron microscope.

RESULTS

Effect of Colchicine on the Distribution of Bound Rh-Con A and on Cell Lysis by Con A

Rh-Con A added to mastocytoma cells at 37°C binds to the cell membrane and is located as a ring form distribution, which remains unchanged throughout the incubation period. This observation is unique, and all these cells bind the lectin in the above-cited form. Incubation of cells with Rh-Con A in the presence of colchicine caused redistribution of Con A membrane sites into "caps." Incubation of control cells with 100 $\mu\text{g/ml}$ Con A at 37°C up to 90 min had no cytolytic effect. Incubation of cells with Con A in the presence of colchicine increased their sensitivity to cytolysis. The effect of the colchicine was dose dependent. The sensitivity of cells to cytolysis by Con A increased with increasing concentrations of colchicine. In parallel, the same concentrations of colchicine increased the number of cells showing caps of Rh-Con A (Fig. 1). Colchicine itself, without Con A, has no cytolytic effect.

Effect of Incubation Time with Con A on Cytolysis and Capping of Cells Treated with Colchicine

To find out the minimum time of incubation with Con A necessary to cause cytolysis, the following experiment was performed. 1.5×10^6 cells were incubated at 37°C with 100 $\mu\text{g/ml}$ of Con A and 10^{-4} M colchicine in PBS from 0 to 90 min

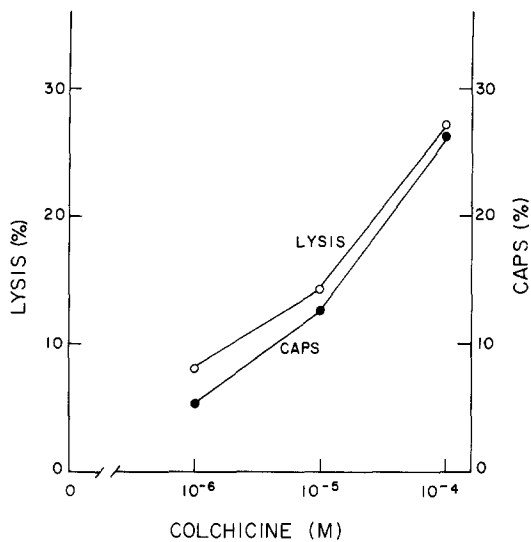


FIGURE 1 Effect of colchicine on the distribution of bound Rh-Con A (●) (tested after 15 min) and on lysis of cells by 100 μ g/ml Con A (○) (tested after 90 min).

with 15-min increments. The cells were then washed with 0.1 M of the hapten inhibitor sugar, α -methyl-D-glucoside, and lysis was determined. Simultaneously, the distribution of Rh-Con A on the cells was tested. The results of such an experiment (Fig. 2) indicate that incubation of colchicine-treated cells for 60 min with Con A lysed 28% of the cells, while at the same time the number of cells showing caps diminished from 28% to 1%.

The lytic process can be prevented by the addition of 0.5–1% of bovine serum albumin (BSA) to the cultures (Fig. 3). It can be seen that the addition of 0.5–1% BSA inhibited lysis of the cells but did not inhibit capping by Con A. Moreover, the presence of caps demonstrates that Con A was present on the cell surface even in the presence of 0.5–1% BSA.

Effect of Low Temperature, Sodium Azide, Glutaraldehyde, and Goat Anti-Con A Antibodies on Capping and Cytolysis

Almost no cap formation or cytolysis occurred in colchicine-treated cells incubated with Con A, at 4°C. Fixation of the cells with 1% glutaraldehyde for 10 min at 37°C, before the addition of Con A, prevented cap formation and decreased lysis to 0.3%. Addition of 10 mM sodium azide to colchicine-treated cells 5 min before the addition

of Con A almost prevented cap formation, and inhibited cytolysis (Table I).

Addition of anti-Con A to cells, after 10-min preincubation with colchicine and Con A, increased cap formation from 28% to 39% tested after an additional 10 min and cytolysis from 30% to 42% tested after 90 min (Table I).

Succinyl-Con A did not induce capping and lysis even in the presence of 10⁻⁴ M colchicine, while the addition of anti-Con A antibodies induced capping and lysis (Table II). The combination of anti-Con A with bound succinyl-Con A exhibited properties similar to those of Con A.

Correlation between Cap Formation and Cytolysis

The percent of cap formation and the percent of cytolysis (data accumulated from 22 experiments) for Con A (Fig. 4) show a linear relationship with a high correlation coefficient (r Con A = 0.98).

Scanning Electron and Fluorescence Microscopy Study of the Cytolytic Process

Interphase mastocytoma cells were round, displaying a rough surface with large numbers of small microvilli (Fig. 5a). Addition of 100 μ g/ml Con A and 10⁻⁴ M colchicine for 5 min did not alter the morphology of the interphase cells. In a parallel fluorescence microscopy study with Rh-Con A, all the cells showed a ring distribution of the fluores-

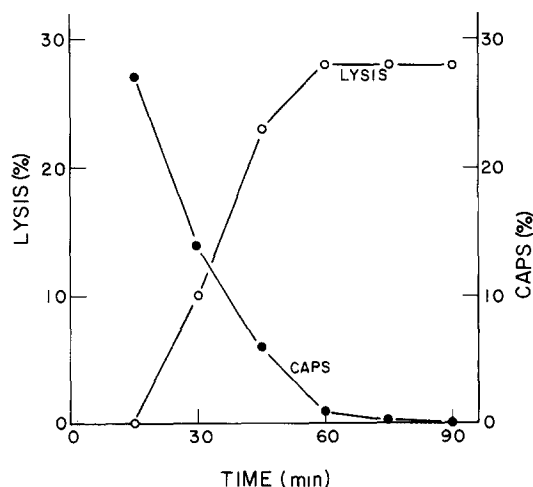


FIGURE 2 Effect of incubation with 100 μ g/ml Con A on cytolysis (○) and on capping (●) of 10⁻⁴ M colchicine-treated mastocytoma cells.

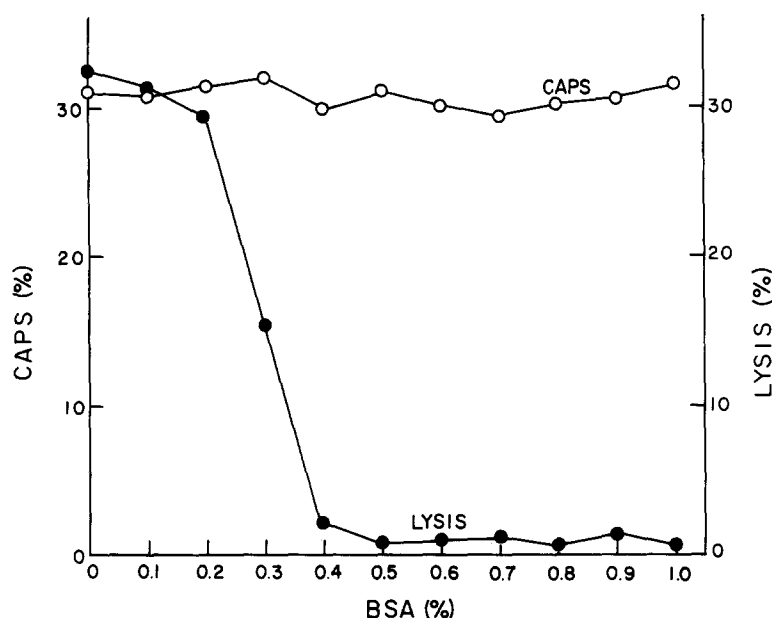


FIGURE 3 Effect of BSA concentration on cap formation (O) and on lysis of the cells (●) induced by 10^{-4} M colchicine and $100 \mu\text{g/ml}$ Con A (tested after 90 min).

TABLE I
Effect of Low Temperature, Sodium Azide, Glutaraldehyde, and Goat Anti-Con A Antibodies on Capping and Cytolysis Induced by Con A on Colchicine-Treated Cells

Treatment*	Caps %	Lysis %
Control	28.7 ± 1.2	30.8 ± 0.8
NaN_3 (10 mM)	0.1 ± 0.1	0.5 ± 0.2
Glutaraldehyde, 1% 4°C	0.9 ± 0.2	0
Normal serum	28.4 ± 1.1	30.1 ± 0.9
‡ Anti Con A (1 mg Ab/ml)	39.2 ± 1.0	42.9 ± 1.1

* Cells were treated with 10^{-4} M colchicine and with $100 \mu\text{g}$ of Con A in addition to the inhibitors and the serum.
‡ Cells preincubated with $100 \mu\text{g/ml}$ Con A plus 10^{-4} M colchicine for 10 min at 37°C ; then 0.1 ml of anti Con A ($100 \mu\text{g/ml}$) was added. Cap formation was measured after 10 min, and lysis was determined after 90 min.

cent lectin in the first 5 min of incubation (Fig. 6a and b). Later on, a small projection began to appear at one point of the cell. This projection displayed a smooth surface almost deprived of surface microvilli (Fig. 5b). With Rh-Con A, it was observed that a small unlabeled projection started to appear at one point of a previously uniformly labeled cell (Fig. 6c and d). After 10-

15 min of incubation, this projection, which was usually connected to the cell through a constriction, progressively enlarged and became smoother and devoid of any clearly defined surface protuberances such as microvilli (Fig. 5c and d). This enlarged and "bare" projection corresponds to the unlabeled part of the cell observed by fluorescence microscopy, while at the other pole of the cell a cap is formed (Fig. 6e and f). After 25 min, the constriction connecting both parts of the cell also

TABLE II
Effect of Succinyl-Con A Plus Anti-Con A on Cap Formation and Cytolysis

Treatment*	Caps %	Lysis %
Succinyl-Con A	0.7 ± 0.1	0.9 ± 0.2
Succinyl-Con A + Colchicine (10^{-4} M)	0.5 ± 0.1	0.8 ± 0.3
Succinyl-Con A + Anti-Con A	0.9 ± 0.6	1.1 ± 0.9
Succinyl-Con A + Colchicine (10^{-4} M) + Anti-Con A	32.3 ± 1.3	36.2 ± 1.9

* Cells were incubated with $100 \mu\text{g/ml}$ succinyl-Con A with or without colchicine (10^{-4} M) at 37°C for 10 min; then $100 \mu\text{g/ml}$ anti-Con A was added. Cap formation was measured after an additional 10 min and lysis was determined after 90 min.

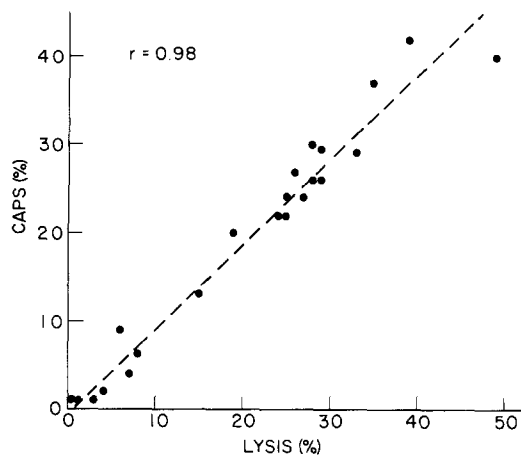


FIGURE 4 Relation between cap formation and cytolysis induced by Con A on colchicine-treated mastocytoma cells. Lysis and cap formation were observed in the same experimental populations which were divided into two parts. One part was treated with Rh-Con A for capping 15 min after the addition of the lectin. The second part was tested 90 min after the addition of Con A for lysis.

became devoid of surface microvilli (Fig. 5e). At the same time, observation of these elongated cells in a fluorescence microscope revealed that the cap was located on the thicker part of the cell (Fig. 6g and h). The percent of the cells displaying such a surface morphology in both SEM and fluorescence microscopy was about 30%. All the remaining cells were spherical and showed a ring distribution of the Rh-Con A and a rough surface with many microvilli.

After 1 h of incubation with Con A plus colchicine, the lytic phase was already in progress, i.e., in SEM the smooth projection now resembled a "spongelike" structure. It seems that at this pole of the cell the destructive lytic effect occurred, while at the opposite side of the cell the microvilli now became shorter and thicker, resembling "nodules"-bleb form (Fig. 7a).

When cell cultures were preincubated with 12 $\mu\text{g/ml}$ lysolecithin for 1 h, they showed a sponge-like structure (Fig. 7c).

Addition of 0.5% of BSA with lysolecithin to cultures at the beginning of the incubation period did not prevent lysis (Fig. 7d). In contrast, when 0.5% of BSA was added to cultures supplemented with Con A and 10^{-4} M colchicine at the beginning of the incubation period, lysis was prevented, the cap-bearing cells remained intact, and the smooth projection was not destroyed (Fig. 7b).

DISCUSSION

The present results show that a linear correlation exists between the lateral mobility of Con A membranal receptors, as indicated by cap formation, and the cytolytic effect of this lectin. It is known that the surface membranes of mammalian cells have been presented as fluid, dynamic structures [Fluid Mosaic Membrane Model] (25). In this model, the fluidity of the membrane allows lateral diffusion of certain membrane components. For example, membrane glycoproteins carrying specific antigenic determinants may rapidly diffuse in the membrane plane (7, 21). During a series of experiments, we demonstrated that cap formation by Con A can be controlled by treatment of the cells with colchicine. Treated with Con A alone, interphase mastocytoma cells do not form a cap. However, when colchicine is added to the cells, they become sensitive to capping by Con A. Increasing the concentration of colchicine is followed by an increase in cap formation and a parallel increase in cell lysis. Thus, interaction of Con A with its membrane receptor sites on mastocytoma cells is shown, in our work, to be modulated by colchicine. Colchicine is known to disrupt microtubules (17, 30, 31, 32), thus enabling modulation of lymphocyte or fibroblast receptor mobility by Con A (3, 6, 27, 34). Our data show that the same phenomenon occurs in mastocytoma cells.

In all experiments, a complete correlation was found between capping and cytolysis. Inhibitors of capping, such as NaN_3 (33), low temperature (19, 33), and fixation of the membrane with glutaraldehyde (19, 22), decreased capping and cytolysis. On the other hand, when antibodies against Con A are added after the binding of the lectin to their receptors on the cell membrane, these multivalent antibodies increase the percent of capping and, in parallel, the percent of cytolysis.

The population which responds to the lectin is about 30% of the interphase cells. This suggests that the sensitivity of the cells to colchicine may be related to a certain phase(s) of the cell cycle. Experiments are now being undertaken to investigate this possibility.

Maximal capping is achieved 15 min after the addition of the lectin. With time, the number of cells with capping decreases in parallel with the increase of the cytolytic effect. Thus, the displacement of the lectin receptors by aggregation into caps at one pole of the cell membrane represents a basic structural change necessary for the occur-

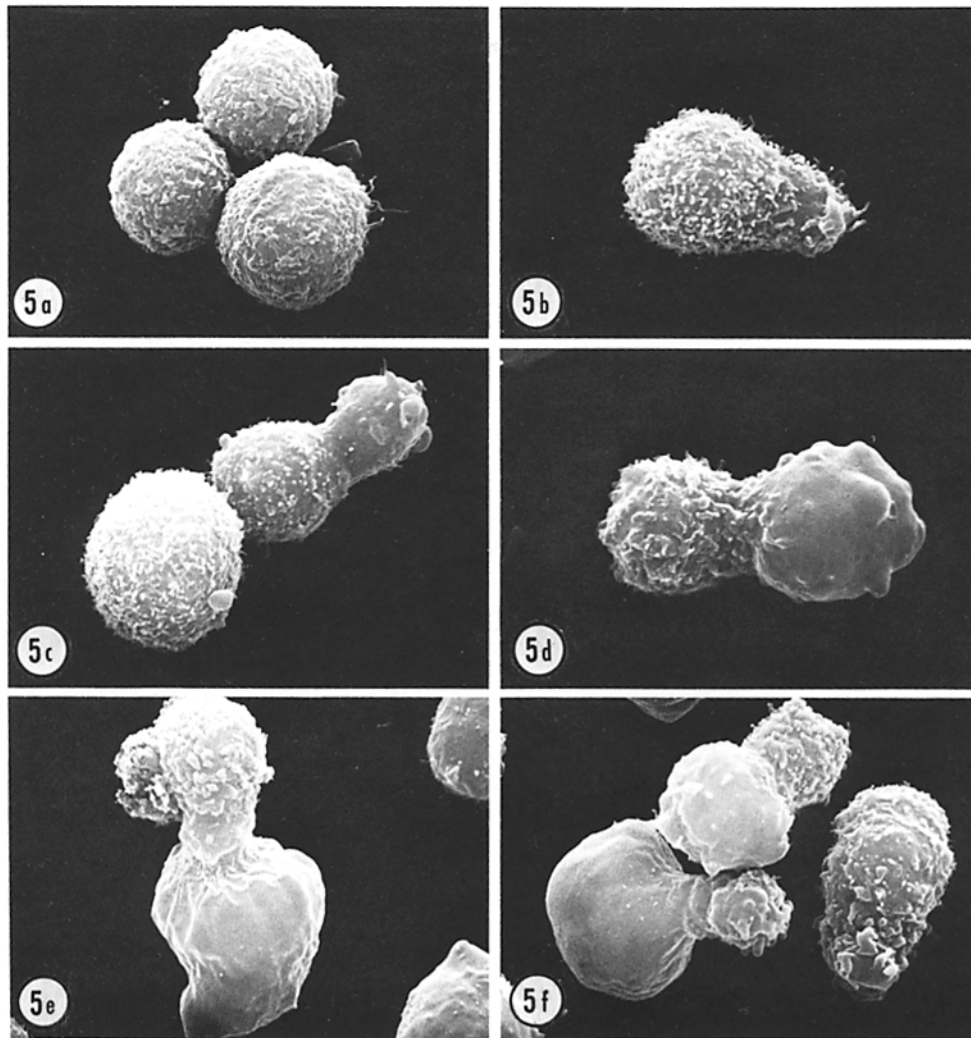


FIGURE 5 (a-f) All illustrations are scanning electron micrographs of P-815-X2 interphase mastocytoma cells. (See Materials and Methods). (a) Round cells displaying a rough surface with many small microvilli (control). $\times 3,800$. (b) A cell from a culture treated with $100 \mu\text{g/ml}$ Con A and 10^{-4} M colchicine for 5 min at 37°C . A small projection starts to form at one point of the cell. This projection displays a smooth surface with a smaller number of surface microvilli. $\times 3,800$. (c-d) Cells were treated with $100 \mu\text{g/ml}$ Con A and 10^{-4} M colchicine for 10 min (c) and 15 min (d) at 37°C . The projection, which is usually connected to the cell through a constriction, progressively enlarges and becomes smoother and devoid of any clearly defined surface protuberances such as microvilli. $\times 3,800$. (e) A cell from a culture treated with $100 \mu\text{g/ml}$ Con A and 10^{-4} M colchicine for 25 min at 37°C . The constriction connecting both parts of the cells becomes devoid of surface microvilli. $\times 3,800$. (f) Cells were treated with $100 \mu\text{g/ml}$ and 10^{-4} M colchicine for 25 min at 37°C . The figure illustrates different stages in the movement of microvilli. $\times 3,800$.

rence of lysis. Such a possibility has recently been suggested by Gingell (8) who showed that membrane permeability changes are due to aggregation of mobile glycoprotein units. In other studies (1,

2), it was shown that fusion or hemolysis of erythrocytes by Sendai virus occurs after viral-induced clustering of membrane glycoproteins and that the areas in which proteins are clustered represent

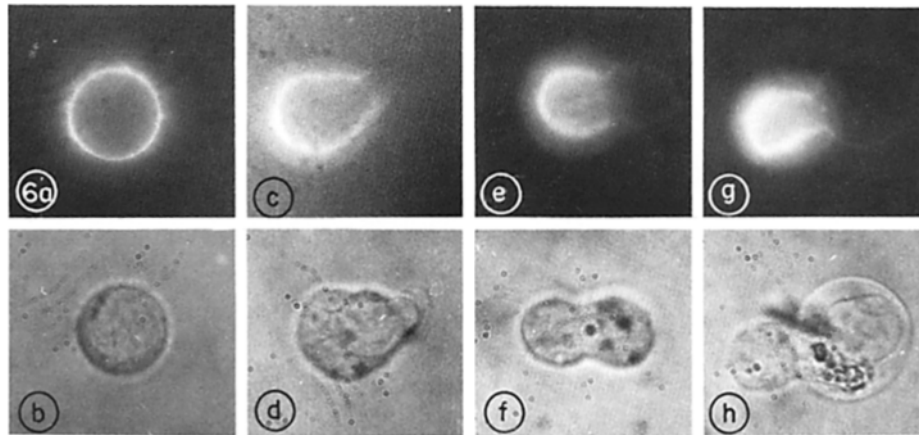


FIGURE 6 (a-h) Fluorescence (a, c, e, and g) and phase-contrast (b, d, f, and h) photographs of interphase mastocytoma cells incubated with 100 $\mu\text{g/ml}$ Rh-Con A and 10^{-4} M colchicine at 37°C . (a and b) A cell after 3 min of incubation. A ring form distribution of Rh-Con A. $\times 1,600$. (c and d) A cell after 5 min of incubation. A small unlabeled uropod starts to form at one point of the labeled cell. $\times 1,600$. (e and f) A cell after 15 min of incubation. Rh-Con A is distributed in a typical "cap" form. $\times 1,600$. (g and h) A cell after 25 min of incubation. The Rh-Con A-labeled cap is separated from the unlabeled part by a constriction. The unlabeled part of the cell begins to swell. $\times 1,600$.

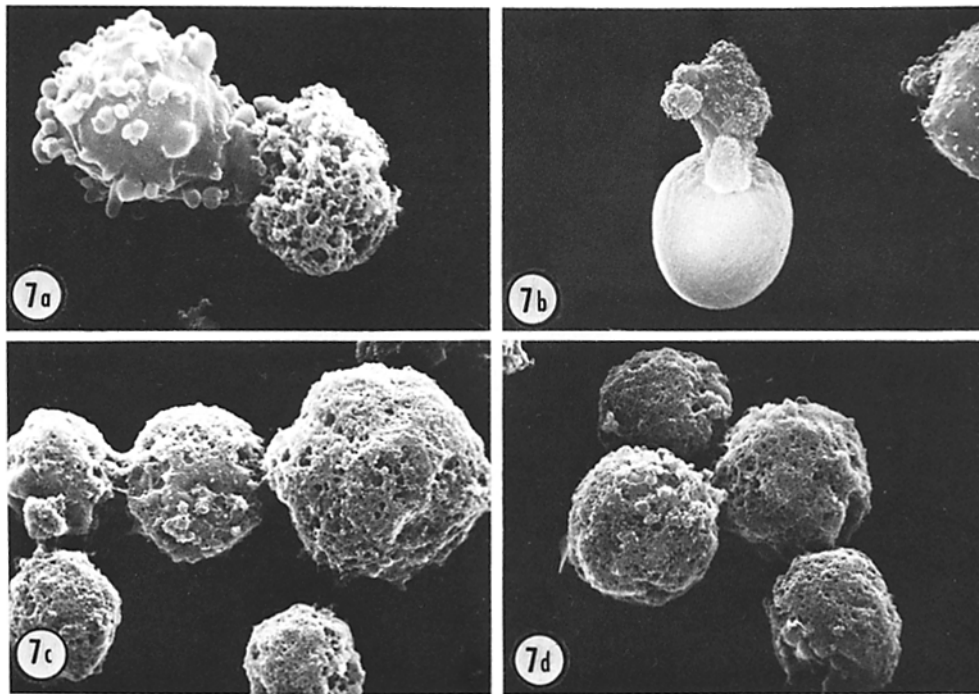


FIGURE 7 (a-d) All illustrations are scanning electron micrographs of interphase mastocytoma cells. (a) A cell from a culture incubated for 1 h at 37°C with 100 $\mu\text{g/ml}$ Con A and 10^{-4} M colchicine. The smooth pseudopod is destroyed and resembles a spongeliike structure. $\times 3,800$. (b) A cell from a culture incubated for 1 h at 37°C with 100 $\mu\text{g/ml}$ Con A and 10^{-4} M colchicine supplemented with 0.5% BSA. The cell is intact, and the smooth pseudopod is not destroyed. $\times 3,800$. (c) Cells incubated for 1 h at 37°C with 12 $\mu\text{g/ml}$ lysolecithin. The cells show a spongeliike structure. $\times 3,800$. (d) Cell incubated for 1 h at 37°C with 12 $\mu\text{g/ml}$ lysolecithin supplemented with 0.5% BSA. The addition of 0.5% BSA has no effect, and the cells show a spongeliike structure. $\times 3,800$.

weakened segments of the membrane through which hemoglobin and ions can escape. These results support our data.

Using a combination of fluorescence microscopy and SEM, we have shown that the microvilli are distributed homogeneously on the resting cell, but that when Con A and colchicine are added the microvilli are concentrated at one pole of the cell, the pole where the cap is formed. Similar observations were previously shown in lymphocytes by DePetris (4), using ferritin-labeled Con A. This author showed, in a transmission electron microscope (TEM) study, that the ferritin-Con A is located by the time the caps are formed, on typical uropods containing microvilli. Van Blitterswijk et al. (29) have also shown, using peroxidase-labeled Con A in a TEM study, that the cap is concentrated over one pole of the cell which shows a villous membrane, while the opposite pole has a smooth, spherical membrane and is virtually free of label. In addition, Unanue and Karnovsky (28) have shown, using SEM, that lymphocytes bearing caps induced by Con A manifest an ameboid form. When colchicine is added to Con A-treated lymphocytes, the number of caps increases in parallel with the number of cells with ameboid features. These authors have shown in SEM micrographs that one pole of such ameboid elongated cells is smooth, while the other (presumably the tail) is rough.

The role of microvilli in surface changes of synchronized mastocytoma cells has been shown by Knutton et al. (12). These authors showed that in G₁ cells most microvilli have a uniform diameter, whereas in S and G₂ cells many microvilli show branching and often originate from much larger surface protuberances. Thus, microvilli confer on cells the ability to stretch without rupture during cytokinesis. The study of mastocytoma cells during the cell cycle by SEM provides evidence for the suggestion that microvilli may be a source of extra surface membrane in a variety of situations. The accumulation and growth of microvilli during the cell cycle of mastocytoma cells may be related to the observed change in fragility to immune and nonimmune lysis which is minimal in later interphase (9, 26). Thus, microvilli confer on cells the ability to stretch without rupture during exposure to hypotonicity or to complement-mediated attack by antibody. These experiments support our interpretation of the results. During the lectin-mediated osmotic lysis (13), the pole of the cell where the microvilli are concentrated remains intact while the opposite pole of the cell which is

smooth and bare of microvilli is destroyed, manifesting a spongelike structure similar to that of cells destroyed by lysolecithin (Fig. 7*b* and *d*). Structural changes in the topography of the cell membrane after different experimental treatments was previously shown by Sheetz et al. in a SEM study (24).

De Petris has previously described a model for cap formation (4). It seems that our results fit this model. Since cap formation is not synchronous, a sample of cells taken after 25 min of incubation includes the different stages of cap formation (Fig. 5*f*). This last observation may explain why the lytic process does not occur abruptly but continues progressively.

From our study with WGA (13), it seems reasonable to suppose that an initial lytic lesion in the trypsin-treated mastocytoma cell membrane accompanies the cap-producing lateral movement of the lectin receptors in the membrane and that the demise of the mastocytoma cell is due to its inability to handle the augmented influx of water into the cell. In that study, we found that use of dextran of high molecular weight, which prevents the influx of water, protects the cell from lysis. In this complementary study with Con A, the microscopic observations (fluorescence microscopy and SEM) have shown that after cap formation the cells start to swell and later burst. The use of 0.5% BSA prevents lysis of the cells (Fig. 7*b*), but does not protect the cells from lysis by lysolecithin (Fig. 7*d*). (In the present study, we could not perform experiments with dextran since it contains the hapten inhibitor sugar of α -methyl-D-glucoside which inhibits Con A.) This differential response suggests that Con A induced osmotic lysis by producing small holes in the membrane such as those caused by antibody and complement (10), while lysolecithin, on the other hand, produced large holes causing widespread damage such as produced by some detergents.

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