THE RELATIONSHIP OF CONCANAVALIN A BINDING TO LECTIN-INITIATED CELL AGGLUTINATION

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ABSTRACT

We have investigated the relationship of concanavalin A binding to the cell surface of normal and transformed cells and the subsequent agglutination of the transformed cells. At room temperature almost no differences could be detected in agglutinin binding between transformed and untransformed cells. At 0°C, however, where endocytosis was negligible, the transformed cells bound three times more agglutinin. However, transformed cells and trypsin-treated normal cells do not agglutinate at 0°C although the amounts of agglutinin bound at 0°C are sufficient to permit agglutination when such cells are shifted up to room temperature. Both transformed and trypsin-treated normal cells show a marked increase in agglutination at 15°C as compared to agglutination at 0°C. From this, as well as the observation that mild glutaraldehyde fixation of the cell surface inhibited agglutination but not agglutinin binding, it was concluded that concanavalin A-mediated cell agglutination requires free movement of the agglutinin receptor sites within the plane of the cell surface.

A number of investigators have employed plant agglutinins (lectins) in the study of the cell surface of normal and transformed tissue culture cells (1–3). It has been demonstrated that many transformed cells and protease-treated normal cells agglutinate at much lower concentrations of agglutinin than do the normal parent cells (3, 4). On the basis of these results, we had proposed a model of the cell surface in which the agglutinin receptor sites of the normal cell were in a conformation which prevented binding of the agglutinin molecules to the cell surface, whereas the agglutinin receptor sites of the transformed cell and the protease-treated normal cell were in a conformation to permit agglutinin binding and thereby initiate agglutination (4).

This hypothesis has been investigated using radiolabeled agglutinin binding assays (5–8). Most of these investigations have demonstrated little or no difference in agglutinin binding between normal and transformed cells. We have recently demonstrated that under carefully controlled conditions, in which nonspecific agglutinin binding and endocytosis of the radiolabeled agglutinin is reduced, transformed cells bind 3.5 times more [^{3}H]concanavalin A than do the normal parent cells (9). We have also shown that concanavalin A binding alone is not sufficient to permit agglutination but that some secondary process has to occur on the cell surface which permits agglutination (9).

Recently Sachs and his collaborators have demonstrated that transformed cells and trypsin-treated normal cells do not agglutinate at 4°C, suggesting that there is a temperature-sensitive step involved in concanavalin A-mediated agglutination (10, 11). Sachs has suggested that this step may be a metabolic step, possibly related to a surface-localized enzymatic activity (10).

Nicolson and Singer have performed an elec-

tron microscope analysis of the pattern of ferritin labeled concanavalin A bound to normal and transformed cells (12–14). This work demonstrates that after concanavalin A binding the pattern of the ferritin label on the normal cell surface is random and homogeneous, whereas on the transformed cell surface the ferritin particles are clumped. Nicolson and Singer have used these data to suggest that the pattern of concanavalin A receptor sites on the normal and transformed cell surface differs before the addition of the lectin. Martinez-Palomo et al. (15) came to a similar conclusion using the peroxidase-diaminobenzidine technique; however, in their study the differences between transformed and normal cells were not uniform and statistical methods were necessary to prove the alterations. Smith and Revel (16) observed no striking differences in the distribution of hemocyanin-labeled concanavalin A between normal and transformed cells, although interesting zonal distributions were found in normal cells. Similar results were also recently obtained by Abercrombie et al. (17).

Thus, agglutination cannot be explained by the simple cross-linking of two cells as a result of increased lectin binding. In order to build a more comprehensive model of agglutination, we have begun investigations into concanavalin A binding to the concanavalin A-specific agglutinin receptor site as well as into the temperature-sensitive process in concanavalin A-mediated agglutination. It should be noted that these studies concern only the mechanism of concanavalin A-mediated agglutination. Wheat germ agglutinin appears to have a somewhat different mode of action.

EXPERIMENTAL

Materials

Jack bean meal, from which concanavalin A was prepared according to the procedures of Agrawal and Goldstein (18), was purchased from Sigma Chemical Co. (St. Louis, Mo.). All tissue culture medium was purchased from Gibco Cultures, Grand Island Biological Co. (Grand Island, N.Y.), except for the sera which were purchased from Baltimore Biological Laboratories (Baltimore, Md.). Tissue culture flasks and dishes were purchased from Falcon Plastics Division of B-D Laboratories, Inc. (Los Angeles, Calif.). [^{3}H]acetic anhydride (500 mCi/mmol) was obtained from Amersham/Searle Corp. (Arlington Heights, Ill.). Aquasol is a product of New England Nuclear (Boston, Mass.). Trypsin and soybean inhibitor were purchased from Sigma Chemical Co. Glutaraldehyde was purchased from Merck Chemical Div., Merck & Co., Inc. (Rahway, N.J.). All laboratory reagents were obtained from either Fisher Scientific Co. (Springfield, N.J.) or Carl Bittmann AG (Basel, Switzerland).

Maintenance of Cell Lines

Both the mouse embryo fibroblast (3T3) cell line and the polyoma-transformed 3T3 cell line (Py3T3) were maintained in Dulbecco modified Eagle's medium + 10% calf serum and 1% penicillin-streptomycin. The 3T3 cell line was always replated before reaching 80% confluency. The cultures were maintained in a moist incubator in which the CO_2 tension was held at 5%.

Each month during the course of the experiments the cell line was tested by autoradiography for pleuropneumonia-like organism (PPLO) contamination and found to be free of mycoplasma contamination at the time of the experiments.

Concanavalin A

[^{3}H]concanavalin A (specific activity 2×10^7 cpm per mg protein) was prepared according to the procedures previously described using [^{3}H]acetic anhydride (9).

Agglutination

Agglutinations were performed as previously described (19). Briefly, 3T3 or Py3T3 cells were grown to 60–80% confluency on Falcon plastic petri dishes (100 mm) in Dulbecco modified Eagle's medium + 10% calf serum and 1% penicillin-streptomycin.

Once the cells had reached the desired density, they were incubated at 37°C ± 1°C on a large warming plate. The cells were washed five times with 5 ml calcium-, magnesium-free phosphate (1 mM KH_2PO_4, 10 mM Na_2HPO_4)-buffered (pH 7.2) 0.154 M NaCl (CMF-PBS) maintained at 37°C and then washed five times with 5 ml calcium-, magnesium-free phosphate-buffered 0.154 M NaCl + 0.02% ethylenediaminetetraacetic acid (CMF-PBS-EDTA)[1] and incubated in 5 ml CMF-PBS-EDTA at 37°C for 15 min. When the cells had rounded up, they were removed from the plate by a gentle washing with 5 ml CMF-PBS-EDTA. The cells were then washed three times with 1 ml phos-

[1] *Abbreviations used in this paper are:* CMF-PBS, calcium-, magnesium-free phosphate-buffered saline; CMF-PBS-EDTA, calcium-, magnesium-free phosphate-buffered saline + 0.02% ethylenediaminetetraacetic acid; PBS, phosphate-buffered saline.

phate-buffered 0.154 M NaCl containing 0.9 mM calcium and 0.4 mM magnesium (PBS).

After washing, the cells were suspended in PBS to a final cell density of approximately 1×10^6 cells per ml. 0.2 ml of the cell suspension was placed in a well of a porcelain spot plate to which 50 μg/ml concanavalin A was added and the preparation was incubated for 15 min at the desired temperature.

After 15 min, a hanging drop slide was prepared and agglutination scored. The percentage of cells agglutinated was determined by counting the total number of cells in a field and the total number of cells in cell clumps of greater than three cells. Three randomly chosen fields within the larger field were counted to obtain the percentage of cells agglutinated. The percentage of agglutinated cells within one field never deviated by more than 10% from the percentage of agglutinated cells in other fields within the same experiment.

Temperature-Dependent Agglutination

In all those experiments in which careful temperature control was required, the agglutinations were performed in a porcelain spot plate suspended in a Haake water bath fitted to a Haake cooling condenser. This system maintained the temperature of the cell suspension at ±0.02°C.

[^{3}H]Concanavalin A Binding

The [^{3}H]concanavalin A binding assay has been more fully described elsewhere (9). Briefly, cells were grown in Falcon plastic tissue culture dishes (35 mm) containing Dulbecco modified Eagle's medium +10% calf serum and 1% penicillin-streptomycin. Once the cells had reached 80% confluency they were incubated at either 0°C or 22°C and washed twice with PBS at the corresponding temperature. The cells were incubated at the desired temperature for 5 min in 2.0 ml phosphate-buffered (pH 7.2) 0.154 M NaCl +1 mM $MgCl_2$ with 50 μg/ml [^{3}H]concanavalin A. The cells were washed five times with 1 ml 0°C or 22°C PBS, precipitated in 1.0 ml trichloroacetic acid, dissolved in 2.0 ml Aquasol, and counted in 10 ml Brays' scintillation solution (20).

The binding data are presented as molecules of concanavalin A bound per μg protein.

Protein Determination

Protein determinations were performed according to the standard Lowry procedure (21) slightly modified for whole cells.

RESULTS

Table I demonstrates that at 22°C, 50 μg/ml concanavalin A will agglutinate 95% of the Py3T3 cells and 100% of the trypsin-treated 3T3 cells. At 0°C, only 10–15% of the Py3T3 or trypsin-treated 3T3 cells are agglutinated by 50 μg/ml concanavalin A. Untreated 3T3 cells demonstrate only 10% agglutination at 0°C or 22°C.

Table I also demonstrates that twice as much [^{3}H]concanavalin A is associated with the cells incubated at 22°C as compared to the cells incubated at 0°C. We have previously demonstrated that this increased "binding" may be the result of endocytosis and nonspecific binding of the concanavalin A molecule at 22°C (9). In order to demonstrate that this increased concanavalin A binding is not responsible for the agglutination observed at 22°C, Py3T3 cells were incubated with 50 μg/ml concanavalin A at 0°C for 15 min. The cells were then washed three times with 0°C PBS to remove the unbound concanavalin A. One-half of the cell suspension was maintained at 0°C while the other half was incubated at 22°C. Agglutination was scored 15 min later for both cell suspensions. As can be seen in Table I, although cells maintained at 0°C demonstrate only 15% agglutination, cells raised to 22°C show the same agglutination as cells which had been maintained at 22°C throughout the agglutination process. This strongly suggests that the excess concanavalin A associated with the cells at 22°C, as compared to cells at 0°C, is not required for agglutination.

Effect of Incubation Time on Agglutination at 0°C and 10°C

Table II demonstrates that the reduction in concanavalin A-mediated agglutination observed at 0°C is not the result of a reduction in the rate of the agglutination process due to a decreased Q_{10}. Incubation of Py3T3 or trypsin-treated 3T3 cells with 50 μg/ml concanavalin A at 0°C for as long as 90 min does not increase the observed agglutination. Similarly, incubation of Py3T3 cells at 10°C for 90 min also produced only 15% agglutination, suggesting that our results are not explained by a high Q_{10} (22).

Rate of Temperature-Dependent Inhibition of Agglutination

Inbar et al. have suggested that the temperature-dependent process in concanavalin A-mediated agglutination might be a metabolic process related to a temperature-sensitive enzyme

TABLE I

Concanavalin A Binding and Agglutination

	Agglutination (%)		Binding (molecules per μg protein)	
	0°C	22°C	0°C	22°C
3T3	10	10	1.5×10^4	1.7×10^5
Py3T3	16	95	4.5×10^4	1.3×10^5
3T3 + 0.001% trypsin for 5 min + 0.005% soybean inhibitor	10	100	3.8×10^4	1.7×10^5
Py3T3 + concanavalin A (0°C), washed three times with 0.154 M NaCl (0°C) and raised to 22°C	—	95	—	4.5×10^4
Py3T3 + concanavalin A (22°C), washed three times with 0.154 M NaCl (22°C)	—	95	—	1.7×10^5

50 μg/ml concanavalin A or [^{3}H]concanavalin A were used in all experiments. Preincubation of the lectin with 1.0 mM α-methyl mannopyranoside for 30 min at 37°C before use prevented all agglutination.

(10). If, instead, concanavalin A-mediated agglutination is dependent on the state of the lipid bilayer, it might be expected that the cessation of agglutination initiated at 22°C but dropped to 0°C would be very rapid. To test this hypothesis, agglutination of Py3T3 cells was initiated at 22°C and agglutination scored every 5 min. Each time an agglutination was scored, a duplicate plate of Py3T3 cells (which has initiated agglutination at 22°C) was dropped to 0°C and agglutination scored 30 min later at 0°C. As can be seen in Table III, once the cells are dropped to 0°C further agglutination is inhibited. This suggests that the inhibition of agglutination at 0°C is very rapid.

TABLE II

Increased Incubation Time and Agglutination at 0°C or 10°C

Time after addition of 50 μg/ml concanavalin A	Agglutination	
	0°C	10°C
min	%	
10	10	12
20	12	11
30	11	15
40	15	10
50	13	16
60	10	13
70	16	15
80	14	12
90	16	15

Agglutinations were initiated at 0°C or 10°C with 50 μg/ml concanavalin A and maintained at 0°C or 10°C throughout the course of the experiment.

Lipid Phase Transitions and Agglutination

It has been suggested that major changes in the fluidity of the lipid bilayer of the plasma membrane occurs at approximately 15°C (23, for a critical analysis of this data see Cone, 22). If the temperature-sensitive step in the agglutination reaction is dependent on the state of the lipid bilayer it might be expected that a change in the pattern of agglutination would be observed at approximately 15°C. Fig. 1 demonstrates that at 15°C there is a rapid increase in the percentage of cells agglutinated, suggesting that agglutination may be related to the state of the lipid bilayer.

Modifiers of Concanavalin A-Induced Agglutination

A number of investigators (24–27) have demonstrated that the addition of multivalent antibodies to lymphocytes or fibroblasts results in a clumping of the antigenic sites on the cell surface. Monovalent antibodies do not produce the observed clumping. It has therefore been suggested that the addition of the multivalent antibodies clumped the antigens which were in a random configuration on the cell surface before the addition of the antibody (24, 25). We have treated Py3T3 and 3T3 cells with a variety of agents which have been demonstrated to prevent clumping and capping of cell surface-localized antigens of both lymphocytes and fibroblasts in order to clarify whether it is the addition of concanavalin A which clumps the agglutinin receptor sites or whether the agglutinin receptor sites are nonrandomly distributed on the cell surface before the addition of concanavalin A.

As we have already demonstrated, Py3T3 and trypsin-treated 3T3 cells do not agglutinate at 0°C. Edidin and Weiss (25) have reported that temperatures below 15°C are the most effective inhibitors of antibody-induced antigen cap formation on fibroblast cell surface.

TABLE III

Rate of Inhibition of Concanavalin A-Initiated Agglutination

Time after initial incubation	Agglutination at 22°C at the indicated time after initiation of agglutination at 22°C	Agglutination at 0°C 30 min after the initial incubation at 22°C
min	%	%
5	39	29
10	47	41
15	60	59
20	73	66
25	85	87
30	90	92

Py3T3 cells were incubated at 22°C with 50 μg/ml concanavalin A. At the given time after the initial incubation, one set of cells was scored for agglutination and a duplicate set of cells were chilled to 0°C and agglutination scored on these cells 30 min after the initial incubation at 22°C.

Mild fixation of the cells with glutaraldehyde would be expected to inhibit lateral movement of proteins in the lipid bilayer. If the concanavalin A receptor sites are clumped before the addition of concanavalin A, mild glutaraldehyde fixation, if it does not inhibit concanavalin A binding, should not inhibit agglutination. However, if the concanavalin A receptor sites clump as the result of the addition of concanavalin A and if this clumping is a prerequisite for agglutination, glutaraldehyde fixation should prevent agglutination. Table IV demonstrates that although Py3T3 cells bind as much concanavalin A after mild glutaraldehyde treatment as they do at 0°C, agglutination does not occur, suggesting that clumping of the receptor sites after the addition of concanavalin A may be necessary to initiate agglutination.

Taylor et al. (24) have demonstrated that preincubation of lymphocytes with metabolic inhibitors or with cytochalasin B prevents capping but does not prevent clumping of the antigens on the cell surface. Table V demonstrates that preincubation of Py3T3 cells with sodium azide, sodium fluoride, sodium cyanide, or cytochalasin

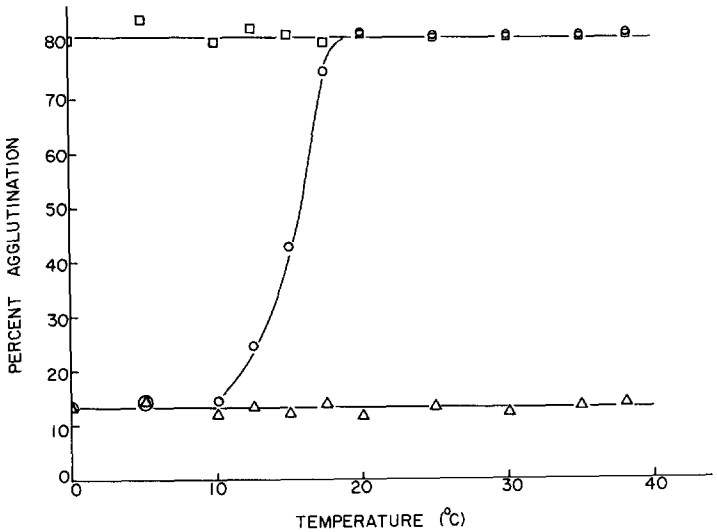

FIGURE 1 Effect of temperature on agglutination. Py3T3 cells were incubated at the indicated temperatures for 15 min with 50 μg/ml concanavalin A. After determination of the percent agglutination at the indicated temperature, the cells were raised to 22°C for 3 min and agglutination was scored at that temperature. At each point a duplicate sample was tested at 0°C. ○—○, agglutination at the indicated temperature; □—□, agglutination of the same cells after having been raised to 22°C for 3 min; △—△, agglutination of a duplicate plate maintained at 0°C for 15 min.

TABLE IV

Effect of Mild Glutaraldehyde Fixation on Agglutination

	Agglutination (%)	Binding (molecules per μg protein)
Py3T3 preincubated with 0.05% glutaraldehyde for 15 min at 37°C, washed three times with PBS	10	4.6×10^4
3T3 preincubated with 0.05% glutaraldehyde for 15 min at 37°C, washed three times with PBS, treated with 0.001% trypsin for 5 min, and 0.005% soybean inhibitor added	10	3.8×10^4
3T3 + 0.001% trypsin for 5 min, 0.005% soybean inhibitor added, and then incubated with 0.05% glutaraldehyde for 15 min.	15	3.8×10^4

All agglutinations and binding were performed at 22°C with 50 μg/ml concanavalin A.

TABLE V

Effect of Metabolic Inhibitors on Agglutination

	Agglutination (%)	Binding, 22°C (molecules per μg protein)
Py3T3 + 10 mM sodium azide for 30 min at 22°C	90	1.2×10^5
Py3T3 + 2.5 mM sodium cyanide and 1.0 mM sodium fluoride for 10 min at 22°C	80	1.3×10^5
Py3T3 + 10 μg/ml cytochalasin B for 1 h	81	1.5×10^5
3T3 + 2.5 mM sodium cyanide and 1.0 mM sodium fluoride for 10 min + 0.001% trypsin for 5 min and 0.005% soybean inhibitor	90	1.8×10^5

All agglutinations and bindings were performed at 22°C with 50 μg/ml concanavalin A.

TABLE VI

Binding and Agglutination after Replating

Hours after replating	Agglutination (%)	Binding, 0°C (molecules per μg protein)
1	18	2.0×10^2
6	25	2.0×10^2
12	21	7.5×10^2
24	20	3.0×10^3
30	35	6.0×10^3
36	56	1.0×10^4
48	80	4.9×10^4

Replating was done with 0.05% trypsin in 0.02% EDTA for 15 min. All agglutinations and binding were done with 50 μg/ml concanavalin A at either 22°C or 0°C.

B does not inhibit agglutination, suggesting that capping may not be necessary for agglutination. When these data are considered with those on glutaraldehyde fixation, it might be suggested that agglutinin receptor site clumping is required for agglutination but that this clumping need not proceed to the point of capping.

Agglutination after Replating

Sachs and his collaborators (28) have previously reported that concanavalin A-mediated agglutination is significantly reduced immediately after subculturing and only returns to its maximal level approximately 48 h after replating. Table VI demonstrates that we have obtained similar results using Py3T3 cells immediately after replating.

Table VI also demonstrates that binding of [^{3}H]concanavalin A to Py3T3 cells after replating does not return to its maximal level until 36–48 h after replating, suggesting that resynthesis of the concanavalin A receptor site after replating is a rather slow process.

DISCUSSION

A number of different theories have been put forth to explain preferential lectin-initiated agglutination of transformed cells.

One of the earliest models was the "cryptic site hypothesis" (4) which suggested that the agglutinin receptor sites are available to agglutinin binding on the transformed cell surface but are buried (cryptic) in the normal cell surface and may be exposed to the agglutinin only by protease treatment. In light of new data (9, 10, 12), this model requires substantial revision.

A second hypothesis, suggested by Sachs and his collaborators, is the "semi-cryptic site hypothesis" (8) which suggests that an equal number of agglutinin receptor sites are exposed on the normal and transformed cell surface but that trypsin-labile substances on the normal cell surface prevent agglutination. Inbar et al. have also demonstrated a temperature-sensitive step in the agglutination process (10) which they considered to be due to a metabolic process.

The third model to explain agglutination is that of Nicolson and Singer (12–14) who could find only a small increase in concanavalin A binding to the transformed cell surface (12) as compared to the normal cell surface. More significantly, they have found that ferritin-labeled concanavalin A demonstrates a clustered distribution on the cell surface of transformed and trypsin-treated normal cells whereas a random, homogeneous pattern is found on the untreated, normal cell surface.

In order to explain concanavalin A-mediated agglutination, we propose the following hypothesis, which takes into account some of the previously published data as well as the data in this paper. In our model, the following steps would have to follow sequentially, if concanavalin A-mediated agglutination of Py3T3 cells is to occur.

(*a*) One active site of a multivalent concanavalin A molecule binds to one of the randomly distributed concanavalin A receptor sites on the transformed cell surface.

(*b*) A second binding site of the same concanavalin A molecule traps a second concanavalin A receptor which is moving around within the same cell surface, thereby initiating the formation of a cluster of the concanavalin A receptor sites. A hydrophobic interaction of the concanavalin A molecule with lipophilic regions of the cell surface may facilitate the binding of the second active site to the second glycoprotein receptor site (9, 29, 30). Anything which could effect the fluidity of the plasma membrane might prevent the movement of the concanavalin A receptor sites within the plane of the surface and thereby inhibit the agglutination process. Such a step could explain the temperature sensitivity of the agglutination reaction, the 15°C transition temperature for agglutination and the effect of glutaraldehyde on agglutination. At this step our model deviates clearly from Nicolson's model (14) in that we would suggest that the concanavalin A molecule is responsible for the clustering of the agglutinin receptor sites while Nicolson would suggest that the receptor sites are clustered before the addition of concanavalin A.

(*c*) Once clustering of the receptor sites has begun, it continues due to the interaction of the multivalent concanavalin A molecule with the multivalent receptor site until the available sites are stably cross-linked by agglutinin molecules. That this process does not continue to the point of cap formation is suggested by the fact that cytochalasin B and metabolic inhibitors do not prevent agglutination although they have been demonstrated to prevent cap formation (22).

In treating the normal cell with trypsin the enzyme might release the concanavalin A receptor sites, permitting them to move in the plane of the membrane. Free mobility of lipids and glycoproteins within the plane of a lipid bilayer have been previously demonstrated (31, 32) and would fit the fluid mosaic model of the plasma membrane (33).

Thus we would suggest that the concanavalin A molecule binds to the randomly distributed, mobile concanavalin A receptor sites on the transformed and trypsin-treated normal cell surface and, due to its multivalency aggregates, the agglutinin receptor sites. Once the cross-links between two cells have increased and taken up a spatial positioning sufficient to overcome the repulsive electrostatic forces between two cells, agglutination will occur. Just how the concanavalin A molecule cross-links two cells is unclear at this time, but the cross-linking might be due to protein-protein or protein-sugar interactions.

The data presented in this paper do not rule out the possibility that transformation or trypsin treatment produces a partial clustering of the agglutinin receptor sites on the cell surface. They do demonstrate that such clustering would be insufficient to allow agglutination, and further clustering, resulting from concanavalin A binding, is necessary to induce agglutination. Preliminary results in our laboratory with fluorescently labeled concanavalin A demonstrate that at 0°C or in the presence of glutaraldehyde no patchy distribution of the agglutinin receptor sites is observed, whereas at 22°C in the absence of glutaraldehyde distinct patches appear. This very strongly suggests that before the addition of concanavalin A the agglutinin receptor sites are arranged in a random distribution.

We would therefore suggest that concanavalin A-mediated cell agglutination, which has previously been considered to be a simple cross-linking of cells by a protein, is in fact a complicated process involving lectin interactions with the lectin specific glycoproteins and free movement of this complex within the lipid bilayer of the plasma membrane.

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