Quantitative Binding of ^{125}I -Concanavalin A to Normal and Transformed Cells

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We have measured the quantitative binding of the radioactively labeled agglutinin ^{125}I -concanavalin A to normal mammalian cells and simian virus 40- and polyoma virus-transformed cells from tissue culture. Parallel measurements of the amount of ^{125}I -concanavalin A necessary to cause agglutination of the cells in suspension were carried out. The transformed and nontransformed cells used for these experiments show large differences in their ability to be agglutinated by ^{125}I -concanavalin A. However, these cell lines have the same number of specific binding sites and similar affinities for the agglutinin whether transformed, trypsinized, or nontransformed. We conclude that the differential capacity of concanavalin A to agglutinate transformed cells relative to normal cells does not result from differences in the number of binding sites between the two types of cells.

A number of proteins, termed phytohemagglutinins because they agglutinate red blood cells, have been isolated from plants (2). Low concentrations of several of these proteins agglutinate cells transformed chemically or by deoxyribonucleic acid (DNA) tumor viruses, whereas agglutination of nontransformed cells requires relatively high concentrations of the proteins (1, 4, 10, 20). The concentration of agglutinin required to agglutinate cells is inversely related to the saturation densities reached in culture (18).

The phytohemagglutinins precipitate different macromolecular carbohydrates and probably interact with the cell surfaced at "exposed" carbohydrate moieties; as expected, the agglutinin activity can be inhibited or even reversed by carbohydrate haptens specific for each phytohemagglutinin (4, 10). Nontransformed cells become agglutinable by the activity of proteases; this observation suggested that nontransformed cells contain "cryptic" binding sites for the phytohemagglutinins and that these sites can be exposed by proteases or by a rearrangement in the architecture of the cell surface by transformation (3, 11).

This hypothesis was based upon quantitative differences in the agglutination of different cell lines (3) and from a report that the amount of ^{63}Ni -labeled concanavalin A (con A) bound to

transformed cells was considerably greater than that bound to nontransformed cells (11). However, the quantitative relationship between the amount of phytohemagglutinin from red kidney beans needed to cause cell agglutination and the amount of the agglutinin bound to the cell surface has been obscure (20). Moreover, in the study with the ^{63}Ni -labeled con A (11), the radioactive marker was not covalently linked to the protein and thus it is difficult to be certain that the determinations of ^{63}Ni binding to the cells actually measured complex formation between the con A and the cell surface; the fact that the amount of ^{63}Ni binding was inhibited less than 50% by an excess of the hapten is particularly unsettling.

We felt that more direct measurements of agglutinin binding to cells and the correlation of that binding to the phenomenon of agglutination were needed to substantiate the "cryptic-site" hypothesis (11). Since con A can be easily purified to physical homogeneity and many of its physical and carbohydrate binding properties are already known (12, 14, 15, 19, 21), it was selected for iodination with ^{125}I (17) to produce a stable covalent linkage between the labeled group and the agglutinin. Although the amount of ^{125}I -labeled con A needed to agglutinate nontransformed cells was much greater than that needed for nontransformed cells, we could not detect any significant difference in the binding of labeled protein to the two types of cells.

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MATERIALS AND METHODS

Tissue culture. Mouse fibroblast cell lines 3T3M-T20 (cloned from a line from R. Dulbecco) and SV-3T3-26-d2 (also from R. Dulbecco) and baby hamster kidney cell lines BHK-21 (original clone from laboratory of M. Stoker) and MT-1 (a polyoma-transformed clone derived from this line) were cultured in plastic petri dishes (Nunc, 100 by 20 mm, or Falcon, 35 by 10 mm) in Dulbecco's modified Eagle's medium (Grand Island Biological Co.) supplemented with 10% calf serum and 25 μg of chlortetracycline per ml.

Con A. Con A was extracted from jack bean meal (Sigma Chemical Co.) and crystallized by the procedure of Sumner and Howell (21). Some preparations were treated with 1 M acetic acid for 20 min and dialyzed to render the protein water-soluble and free from any carbohydrates (14). All preparations were adsorbed to a Sephadex G150 column and eluted with buffers at low pH as described by Olson and Liener (14).

^{125}I -labeled Con A. Specific iodination of tyrosine residues with lactoperoxidase (the kind gift of M. Morrison) was carried out as described by Phillips and Morrison (17) with modifications of concentrations as follows. The con A at a tetramer concentration of 10^{-4} M to 1.6×10^{-4} M was dissolved in 0.1 M sodium phosphate buffer (pH 7.4) containing Na^{125}I at 4×10^{-4} to 4×10^{-3} M (specific activity, 200 to 500 $\mu\text{Ci}/\mu\text{mole}$). The reaction was initiated by the addition of lactoperoxidase to a concentration of 7.7×10^{-7} M and hydrogen peroxide at 8×10^{-5} to 1.6×10^{-4} M. The incorporation of radioactivity was followed by precipitation of the protein with 5% trichloroacetic acid for counting.

^{125}I counting. A Nuclear-Chicago model 1085 gamma counter was used for determining the ^{125}I radioactivity.

Agglutination assays. Cell agglutination was assayed by a modification of the procedure described by Burger and Noonan (5). Subconfluent cells were removed from the plates with 4×10^{-4} M ethylenediaminetetraacetic acid (EDTA) in buffered saline at 37 C and washed once with buffered saline containing Mg^{2+} and Ca^{2+} before being washed and suspended in buffered saline lacking Mg^{2+} and Ca^{2+} . A 100- μl solution of 2×10^5 to 10^6 cells/ml was mixed with an equal sample of various concentrations of agglutinin dissolved in buffered saline in the wells of a porcelain spot plate. The plate was kept in gentle motion at 22 C, and samples of 20 to 50 μl were removed at 5, 10, and 30 min and observed in the wells of plastic spot plates (Linbro Chemicals FB-48) with an inverted microscope.

Binding assays. Method 1 was as follows. Subconfluent cells were removed from plates with 4×10^{-4} M EDTA in buffered saline lacking Mg^{2+} and Ca^{2+} , washed once with buffered saline containing Mg^{2+} and Ca^{2+} and then washed and suspended in buffered saline without Mg^{2+} and Ca^{2+} at a density of 5×10^5 to 10^6 cells/ml. Different concentrations of ^{125}I -labeled con A with or without 0.3 M α -methyl-D-glucopyranoside, a hapten for con A, were added, and the cells were incubated with slow agitation for 15 min at 0 C. After

centrifugation at $700 \times g$ for 1 min, the cell pellet was washed two times with cold buffered saline and then solubilized with 0.5 N NaOH; a sample was counted directly in a gamma counter and assayed for total protein by the method of Lowry et al. (13).

Method 2 was as follows. The cells remained attached to the culture plates. Subconfluent cells on 35-mm plastic petri plates were washed two times with cold buffered saline lacking Mg^{2+} and Ca^{2+} and then were incubated with 0.3 ml of a solution containing different concentrations of ^{125}I -con A with and without hapten. After 15 min at 0 C, the liquid was aspirated and the plates were washed twice with cold buffered saline. After the cells were solubilized in 0.5 N NaOH, they were analyzed as mentioned above. Control plates incubated without agglutinin were trypsinized, and the cells were counted in a hemocytometer.

RESULTS

Radioactive labeling of con A. After reaction of con A with ^{125}I in the presence of lactoperoxidase and peroxide, the protein was dialyzed and then adsorbed to a Sephadex G150 column. Figure 1 shows the elution profile of the radioactivity. Acrylamide gels of the material specifically bound to and eluted from Sephadex gave only one peak in the normal position for purified con A. From the specific activity of the iodine, it was determined that one residue of tyrosine per 27,000 molecular weight had been iodinated. This molecular weight is assumed to be the intact monomer (22); however, at the pH of the binding studies,

FIG. 1. Dextran adsorption and elution of 5 mg of ^{125}I -con A (acetic acid-treated). The protein was adsorbed to a column (1 by 27 cm) of G-150 Sephadex in 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 7.2) containing 10^{-3} M CaCl_2 and 10^{-3} M MgCl_2 . The effluent was monitored for ^{125}I radioactivity in a gamma counter. Adsorbed material was eluted with 0.02 M glycine-HCl (pH 2.0) and dropped into tubes containing 0.075 M Tris-hydrochloride (pH 9.0) to bring the solution to neutrality.

the con A probably exists as a tetramer (15, 21, 22).

The ^{125}I -con A retained its hapten properties as demonstrated by its ability to be adsorbed onto Sephadex and eluted by either 0.1 M glucose or low pH and by the fact that the binding to cells can be competed for or reversed by α -methyl-D-glucopyranoside but not by *N*-acetylglucosamine. Competition experiments were done by binding ^{125}I -con A to cells in the presence of cold, unmodified con A. All of the ^{125}I -con A was competed by unmodified con A, and the competition experiments gave the same binding constants for ^{125}I -con A as unmodified con A.

Agglutination of transformed and nontransformed cells. Table 1 gives the levels of ^{125}I -con A and unlabeled con A required to obtain agglutination of mouse fibroblasts [3T3 M and SV-3T3 (simian virus 40-transformed) or 3T6 (non-viral-transformed)] and baby hamster kidney cells [BHK-21 (nontransformed) or MA-8 (abortively transformed) and MT-1 (polyoma virus-transformed)] and the levels required after mild trypsinization.

TABLE 1. Levels of ^{125}I -con A or unlabeled con A required for agglutination of mouse fibroblasts and baby hamster kidney cells

Cell line	Concn of ^{125}I -con A or unlabeled con A ($\mu\text{g/ml}$) for half-maximal agglutination
Mouse	
Normal	
3T3M.....	70-120
3T3M after 0.01% trypsin for 10 min.....	7
Transformed	
SV3T3.....	7-15
SV3T3 after 0.01% trypsin for 10 min.....	7
3T6.....	7-10
3T6 after 0.01% trypsin for 10 min.....	7-10
Hamster	
Normal	
BHK-21.....	25-30
BHK-21 after 0.01% trypsin for 10 min.....	7
MA-8.....	100-120
MA-8 after 0.01% trypsin for 10 min.....	10
Polyoma virus-transformed	
MT-1.....	7
MT-1 after 0.01% trypsin for 10 min.....	7-10

FIG. 2. Binding of ^{125}I -con A to cells in solution by method 1. (A) 3T3M cells (10^5) at 0 C; (B) SV3T3 cells (10^5) at 0 C. Symbols: X, untreated cells plus ^{125}I -con A, counts per minute bound; □, cells treated with 0.01% trypsin for 10 min plus ^{125}I -con A, counts per minute bound; Δ, cells plus 0.3 M α -methylglucopyranoside plus ^{125}I -con A, counts per minute bound.

The transformed and nontransformed cells used for these experiments exhibited significant differences in their ability to be agglutinated by con A. The same differences were observed for wheat germ agglutinin activity with these cells (*unpublished data*), and they confirm the differences seen in agglutinability by others (1, 3, 4, 10, 20). However, these cell lines have the same number of con A binding sites available whether transformed, trypsinized, or nontransformed as measured by the specific binding of ^{125}I -labeled con A, calculated on the basis of equal concentrations of cell protein, or have a slightly greater number of binding sites for nontransformed cells compared to transformed or trypsinized cells calculated on the basis of equal cell numbers.

Binding of ^{125}I -concanavalin A to transformed and nontransformed cells. Figure 2 shows typical binding curves for ^{125}I -con A to 3T3 and SV-3T3 cells in solution. The half-maximal binding of

TABLE 2. Half-maximal binding of ^{125}I -con A to 3T3 versus SV-3T3 and BHK versus polyoma-BHK cells

Cell line	Cell number	Method	Temp of binding	Concn of ^{125}I -con A ($\mu\text{g/ml}$) for half-maximal binding
Mouse				
Normal				
3T3M.....	5×10^5	1	22	40-50
3T3M.....	5×10^5	1	0	60-80
3T3M.....	10^5	1	0	60-80
3T3M, 0.01% trypsin for 10 min.....	10^5	1	0	60
3T3M.....	3×10^5	2	0	75
3T3M.....	10^5	2	0	50-60
Simian virus 40-transformed				
SV3T3.....	5×10^5	1	22	40
SV3T3.....	5×10^5	1	0	60-80
SV3T3.....	10^5	1	0	60-80
SV3T3, 0.01% trypsin for 10 min....	10^5	1	0	60-80
SV3T3.....	3×10^5	2	0	60-80
SV3T3.....	10^5	2	0	50
Hamster				
Normal				
BHK, MA-8.....	2×10^5	1	22	30
BHK, MA-8.....	10^5	1	0	35-40
Polyoma virus-transformed				
Polyoma-BHK, MT-1.....	2×10^5	1	22	40
Polyoma-BHK, MT-1.....	10^5	1	0	35-40

^{125}I -con A (at different cell concentrations, at temperatures of 22 or 0 C, with or without mild trypsin digestion) to 3T3 versus SV-3T3 and BHK versus polyoma-BHK cells is given in Table 2.

Since proteolytic digestion of cells resulted in greater agglutinability, it was conceivable that small amounts of proteolytic enzyme released from the cells when they were removed from the plates could cause erroneous interpretation of the specific binding results. To overcome this problem, the binding assay was done with cells still attached to the plates as well as with the cells in solution. Typical binding curves for ^{125}I -con A to 3T3M and SV-3T3 cells at 10^5 cells per 35-mm plate are given in Fig. 3 and reveal the same number of sites per cell for normal or transformed cells, $\sim 10^7$ to 3×10^7 . The half maximal binding of ^{125}I -con A to these cell lines and to BHK normal and polyoma-transformed cell lines is constant for 3×10^4 to 10^5 cells per 35-mm plate and occurs at a con A concentration of $\sim 70 \mu\text{g/ml}$; the binding is inhibited by 0.03 M α -methyl-D-glucopyranoside and is completely competed for by unmodified con A. No difference in the amount of binding to normal and transformed cells was observed for con A labeled with ^{14}C -iodoacetamide.

DISCUSSION

Our data indicate that there is an equal number of con A binding sites on the cell surface of trans-

formed and nontransformed cells. Therefore, the differential capacity of con A to agglutinate transformed cells relative to nontransformed cells does not result from differences in the number of binding sites between the two types of cells.

If there is indeed no difference in the number of con A binding sites or indeed of other agglutinin-binding sites (20) between transformed and nontransformed cells, then how can one explain the differential agglutinability of the cell lines? Three models can be generated to explain the phenomenon: (i) steric interference, (ii) allosteric conversion, and (iii) ionic interaction. Steric interference would require that the agglutinin-binding site be buried on the surface of the normal, non-transformed cell, such that the second hapten binding site of the agglutinin is unavailable for binding to a site on an adjacent cell. Conversely, the transformed cell binding site would be more external, allowing the second hapten binding of the agglutinin site to attach to another cell.

Allosteric conversion would require that the binding of agglutinin to the transformed cell cause a rearrangement in the structure of the surface, such that the lectin is able to interact with adjacent cells, or that the binding of agglutinin to the nontransformed cell cause a rearrangement in the cell surface such that the second hapten site on the agglutinin cannot participate in second binding interactions with another cell, or both.

FIG. 3. Binding of ^{125}I -con A to cells by method 2. (A) 3T3M cells (10^6) at 0 C; (B) SV3T3 cells (10^6) at 0 C. Symbols: X, cells plus ^{125}I -con A, counts per minute bound; Δ , cells plus 0.03 M α -methylglucopyranoside plus ^{125}I -con A, counts per minute bound.

The argument of ionic interaction suggests that the ability of cells to grow on top of other cells or to be contact-inhibited by the presence of adjacent cells and the ability of cells to adhere to one another in the presence of copolymers (7) or specific proteins may both depend on the configuration and localized charge which cells "see" on adjacent cells. It is possible that the strong membrane potential common to cells (8, 9) is ionically masked on the surface of the transformed cell. This model requires no difference in number of binding sites for agglutinins on transformed and normal cells but simply a difference in the charge repulsion of the cells. The digestion of cell surfaces by proteolytic enzymes does not alter the amount of specific binding of con A as we described above but could drastically alter the ionic makeup of the cells. Indeed, some levels of trypsinization alone can cause cells to become sticky and adhere to one another without the presence of external proteins, whereas other de-

grees of trypsinization cause the cells to become monodisperse.

Although we do not know enough about the actual differences in cell surfaces between transformed and normal cells to distinguish clearly a mechanism for agglutination, we have shown that transformation does not create or uncover new binding sites for the agglutinin con A, nor has such a difference been observed for the phytohemagglutinin from red kidney beans; perhaps no difference exists for any of the agglutinins. The models which can explain such observations require more sophisticated understanding of the interaction of cell surfaces and their structural makeup.

Similar results for the specific binding of lectins to transformed and normal cells have been found concurrently in other laboratories (6, 16).

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