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Agglutination of Sindbis Virus and of Cells Infected with Sindbis Virus by Plant Lectins

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We have examined the agglutination of Sindbis virus and of chick and hamster cells infected with Sindbis virus by two of the plant lectins, concanavalin A and *Ricinus communis* agglutinin. Both lectins agglutinate the virus by binding to the polysaccharide chains of the envelope glycoproteins. Both chick and hamster cells exhibit increased agglutination by the lectins after infection by Sindbis virus. In the case of chick cells infected with Sindbis virus, this increase in agglutinability occurs between 3 and 5 h after infection. Infected and mock-infected cells bind the same amount of ^{3}H-labeled concanavalin A, which suggests that the increase in agglutination after infection is due to rearrangements at the cell surface rather than to insertion of new lectin binding sites per se.

It is clear from work done in several laboratories that cells transformed by tumor viruses and carcinogens or treated lightly with proteases are agglutinated by much smaller amounts of the plant lectins than is required to agglutinate normal (untreated) cells (9, 13, 15, 16, 26). Most of the recent experimentation has been done with four of the lectins: (i) concanavalin A (Con A), which binds to α-D-glucose and α-D-mannose-like sites (2); (ii) wheat germ agglutinin, which binds to *N*-acetyl-D-glucosamine-like sites (9); (iii) soy bean agglutinin, which binds to *N*-acetyl-D-galactosamine and α-D-galactose-like sites (26); (iv) *Ricinus communis* agglutinin (RCA), which binds to α-D-galactose and L-rhamnose-like sites (19). The mechanism for this change in agglutination appears to be a rearrangement or clustering of binding sites, since transformed cells bind the same amount of radioactively-labeled Con A (4, 10, 21) and wheat germ agglutinin (21) as normal cells, indicating that increased agglutination is not due to an increase in the total number of binding sites. Moreover, electron microscope studies with ferritin-conjugated Con A show a clustering of the conjugate on the surface of transformed cells, as opposed to the uniform distribution of Con A sites on normal cell surfaces (18).

Similar changes in agglutination have also been observed in cells infected by enveloped viruses (5, 23). We have been studying the agglutination of the Sindbis virion, a group A arbovirus, and of cells infected with Sindbis virus.

MATERIALS AND METHODS

Lectins. Con A was purchased from Miles-Yeda, 3X crystallized, or purified by the method of Agrawal and Goldstein (2). RCA was a gift of G. L. Nicolson.

Agglutination of Sindbis virus. Purified Sindbis virus, grown in chicken embryo fibroblasts and labeled with ^{14}C-methionine (28, 29) was dialyzed extensively at 4 C against phosphate-buffered saline, pH 7.4 (PBS of Dulbecco and Vogt [12] but lacking Ca and Mg). After dialysis the protein concentration was 60 μg/ml, as determined by the method of Lowry et al. (17). Bovine serum albumin was then added to a final concentration of 1 mg/ml to stabilize the virus. To 0.8 ml of the virus suspension (2,500 counts/min) was added 0.2 ml of various concentrations of Con A and RCA in PBS; after 2 h at room temperature, the reaction mixtures were centrifuged at 1,000 × *g* for 15 min at 4 C, and half of the supernatant fluid was assayed for radioactivity. The precipitates were washed once and then dissociated by a 0.5-ml wash of 0.1 M α-methyl-D-mannoside or 0.2 M α-D-galactose, respectively. After centrifugation to remove any remaining aggregates, the solubilized precipitates were assayed for radioactivity.

Agglutination of cells. The agglutination procedure used followed closely that of Sela et al. (26). The cells were removed from the petri plates with a solution of 1 mM EDTA in PBS and were washed three times in PBS. The cells were filtered through 20-μm nylon mesh to eliminate clumps of cells, and the concentration was adjusted to 10^6/ml. Various concentrations of the lectins in 0.1 ml were mixed with an equal volume of cells in Disposo-trays (Lindbro). This suspension was incubated at room temperature for 30 min and was then scored for agglutination using an inverted phase microscope, with half-maximal agglutination defined as in Eckhart et al. (13).

Cell infection. Confluent monolayers of chicken embryo fibroblasts or of BHK-21 cells in 60-mm petri plates were infected with 50 plaque-

forming units per cell at 37 C in 0.5 ml of PBS (with 0.9 mM Ca^{2+} and 0.5 mM Mg^{2+}) containing 1% fetal calf serum and 1 μg of actinomycin D per ml. One hour after infection the inoculum was removed, the plates were washed, and 3 ml of Eagle minimal essential medium containing 3% fetal calf serum and 1 μg of actinomycin D per ml was added. Two hours after infection the medium was changed to medium lacking actinomycin D. Mock-infected cells were treated identically, except without virus.

The use of actinomycin D improves the virus yield, presumably due to suppression of interferon production. However, virus stocks stored at −50 C in Eagle medium containing 3 to 5% fetal calf serum inactivate to some extent if actinomycin D is present, whereas virus stocks are stable under these conditions in the absence of actinomycin D. Thus, actinomycin D is routinely used only in the early stages of virus infection.

Preparation of labeled Con A. ^{3}H-Con A was prepared by the acetylation procedure of Agrawal et al. (3) using ^{3}H-acetic anhydride (specific activity 50 mCi/mmol), except that 0.1 M α-methyl-D-mannoside was present during the reaction to protect the binding sites. ^{3}H-Con A was identical to native Con A in migration on a Sephadex G-100 column in 0.2 M sucrose and in agglutination of cells; also, native Con A competed with ^{3}H-Con A for binding to cells. The specific activity of the ^{3}H-Con A used for binding experiments was 165 counts per min per μg.

Binding of labeled Con A to cells. Monolayers of chick cells (in 60-mm petri plates), either infected or uninfected, were washed three times with PBS and then various amounts of ^{3}H-Con A in 1 ml of PBS were added. Control plates were treated with ^{3}H-Con A in the presence of 0.1 M α-methyl-D-mannoside. After incubating for 45 min at room temperature, the monolayers were washed three times with PBS and then dissolved in 2 ml of 1% sodium dodecyl sulfate (SDS); 0.2 ml of each dissolved monolayer was assayed for radioactivity. There was an average of 3.5×10^6 cells per monolayer, determined by trypsinizing several mock-infected and infected monolayers and counting them in a hemocytometer.

Pronase digestion and affinity chromatography. Sindbis virus, labeled with radioactive glucosamine or fucose, was prepared as previously described (28, 29). Under the conditions used, greater than 97% of these labels migrated with the membrane glycoproteins of the virus on SDS-polyacrylamide gels, and greater than 90% remains in the original chemical form (28). Polysaccharide chains from the glycoproteins were prepared by Pronase digestion of the virus (8) (>95% of the label rendered acid-soluble) followed by dialysis against PBS. The polysaccharides were then tested for adsorption on a Con A-Sepharose 4B affinity column, prepared by the method of Cuatrecasas and Anfinsen (11). The Con A on the column was biologically active in that ^{14}C-glycogen could be adsorbed to the column and eluted with 0.1 M α-methyl-D-mannoside. Columns containing approximately 1 ml of Con A-Sepharose were poured at room temperature and washed extensively with PBS. Digests of either ^{3}H-glucosamine- or ^{3}H-fucose-labeled Sindbis virus in 0.5 ml of PBS were then applied and allowed to equilibrate with the column for 2 h at room temperature. The column was washed with PBS until no label was detected in the effluent and finally eluted with 0.1 M α-methyl-D-mannoside.

RESULTS

Agglutination of Sindbis virus by Con A and RCA. Since it has been shown that Con A binds to Semliki Forest virus (5, 20), we expected that Con A and perhaps other lectins would bind to and agglutinate Sindbis virus. The results in Table 1 indicate that both Con A and RCA agglutinate Sindbis virus. No agglutination is observed in the presence of 0.1 M α-methyl-D-mannoside for Con A or 0.2 M α-D-galactose for RCA, and Sindbis virus-lectin precipitates are dissociated by the appropriate inhibitor. Thus, the agglutination is specific and reversible. We have also found that wheat germ agglutinin will agglutinate the virus.

To test if the Con A was bound through the envelope glycoproteins of Sindbis virus (28), the polysaccharide chains of the virus were prepared by Pronase digestion. These polysaccharides, labeled with either ^{3}H-glucosamine or ^{3}H-fucose, were tested for binding to a Con A-Sepharose 4B affinity column. For either label, about 75% of the total label added was adsorbed to the

TABLE 1. *Agglutination of Sindbis virus with Con A and RCA*

Con A			RCA		
Concentration (μg/ml)	Counts/min (% of total)		Concentration (μg/ml)	Counts/min (% of total)	
	Supernatant	Precipitate after dissociation		Supernatant	Precipitate after dissociation
0	100	—[a]	0	100	—
5	95	—	1.5	50	42
10	96	—	3	11	85
25	41	55	6	4.5	91
50	7.5	88	10	3.2	90
75	2.6	91	25	1.7	93
100	2.3	90	50	1.3	92
150	1.5	92	100	0.6	94
150[b]	100	—	100[c]	100	—

[a] No precipitate.
[b] Contained 0.1 M α-methyl-D-mannoside.
[c] Contained 0.2 M α-D-galactose.

columns; all of the bound label could be eluted with 0.1 M α-methyl-D-mannoside. Most of the nonadsorbed label could be adsorbed if applied to another Con A-Sepharose column, and in this way over 95% of the total label could be adsorbed. No label was adsorbed to a column of Sepharose 4B alone, and 0.1 M *N*-acetyl-D-glucosamine could not elute any of the label adsorbed to the Con A-Sepharose columns.

The envelope glycoproteins of Sindbis virus contain at least 3 polysaccharide chains which differ in length and composition (8). From the previous results, it appears that all three chains are bound by Con A. As a confirmation of this, the various polysaccharide chains were separated by Biogel P-6 chromatography before and after adsorption to Con A-Sepharose columns (Fig. 1). In each panel of Fig. 1, the ^{14}C-glucosamine-labeled polysaccharide chains (open points) have not seen Con A and are used as a reference. As is shown in panels (a) and (b), polysaccharide chains labeled with ^{3}H-glucosamine migrate identically with the marker regardless of whether the label is present in a total digest (Fig. 1a) or if the label is present in chains specifically bound to Con A (Fig. 1b). Similarly, no difference in pattern is detectable for fucose-labeled chains after specific adsorption to Con A columns (Fig. 1c, d). Thus, all three classes of the polysaccharide chains of the envelope glycoprotein of the virus are bound specifically by Con A.

Agglutination of infected cells. Figure 2 shows the agglutination of chicken embryo fibroblasts at various times after infection by Sindbis virus. Between 3 and 4 h after infection, when approximately 1 plaque-forming unit per cell has been released, infected cells begin to exhibit increased agglutination with Con A and RCA. Agglutinability of the cells continues to rise until 5 h after infection and then levels off. At 5 h after infection, approximately 100 plaque-forming units per cell have been released, or about 1% of the final virus yield (Fig. 2). No agglutination was ever observed in control incubations containing the inhibitor α-methyl-D-mannoside or α-D-galactose for Con A and RCA, respectively. The same result was obtained when hamster cells (BHK-21) were infected with Sindbis virus, except that the change in agglutinability begins about 5 h after infection and is complete at 6 h. These results do not agree with those of Becht et al. (5), who did not see a consistent increase in agglutination of chick cells after infection with Sindbis virus but did see an increase in the agglutination of hamster cells after Sindbis virus infection.

Binding of ^{3}H-Con A to infected and mock-infected cells. Figure 3 shows the amount of ^{3}H-Con A bound to infected and uninfected cell monolayers when various amounts of the lectin are added. The control incubations (dotted lines) were identical except for the presence of 0.1 M α-methyl-D-mannoside. Mock-infected cells (solid points) appeared to bind slightly more Con A than infected cells, but this difference appears to be due to nonspecific adsorption, since uninfected cells also bind more Con A in the presence of the specific inhibitor. In this experiment the cell monolayers had been infected or mock-infected for 7 h. At this point in infection, approximately 400 plaque-forming units of Sindbis virus have been released per cell (Fig. 2), but the monolayers show only minimal cytopathic effect. From this experiment we can conclude that the marked increase in agglutinability of cells after infection is not due to an increase in the total number of available binding sites.

The number of Con A molecules specifically bound per cell was calculated from the data in Fig. 3, using the specific activity of the ^{3}H-Con A (165 counts per min per μg), the number of cells per plate (3.5×10^{6}), and assuming a molecular weight of 10^{5} for Con A (24). Each cell, whether infected or uninfected, binds 1.5×10^{7} molecules of Con A. This value agrees reasonably well with values previously obtained with other cells (4, 21).

DISCUSSION

We have shown that Sindbis virus is agglutinated by several of the plant lectins, including Con A, RCA, and wheat germ agglutinin, and that this agglutination is effected through polysaccharide chains covalently linked to the surface (glyco) proteins of the virion. It was somewhat surprising to us that Con A would attach to all of the polysaccharide chains of the virion, because Con A is reported to bind specifically to a terminal mannose, glucose, or glucosamine (14, 27). The specificity of the other lectins has not been explored in as much detail, but if RCA and wheat germ agglutinin also bind to terminal carbohydrates, the virus polysaccharides must exhibit a high degree of branching. We note that the larger virus polysaccharides also contain fucose and sialic acid which are always terminal. Thus these chains, no greater than 16 to 18 residues in length, may possess five or more termini.

After infection of cells by Sindbis virus or other group A arboviruses, the two envelope glycoproteins (25, 29) of the virion are synthesized de novo and inserted into the cell surface; the virus nucleocapsid buds only through regions

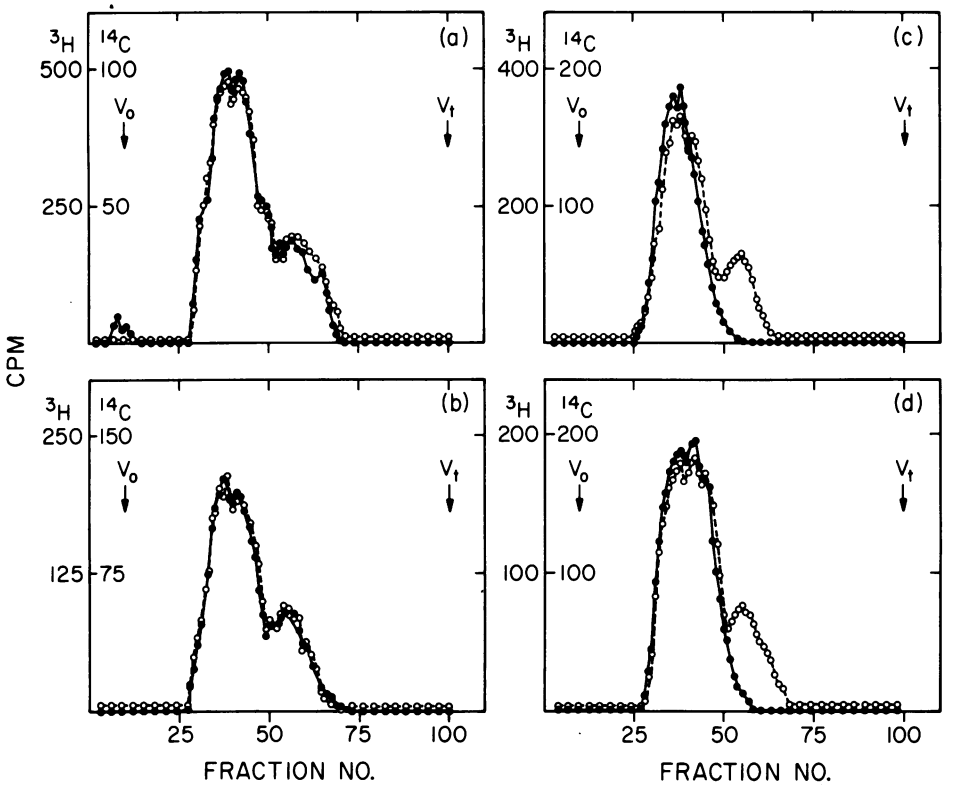

FIG. 1. *Polysaccharide chains of Sindbis virus on a Biogel P-6 column. Pronase digests of Sindbis virus, labeled with ^3H-fucose, ^3H-glucosamine, or ^{14}C-glucosamine were applied to a 0.9- by 60-cm column of Biogel P-6, equilibrated with 0.1 M phosphate (pH 7.8) containing 0.1% SDS. Ten milliliters of effluent was collected into a graduate cylinder, and then 0.22-ml fractions were collected directly into liquid scintillation vials. Digests of ^{14}C-glucosamine-Sindbis virus which were not adsorbed to Con A. Sepharose columns were used as markers. (a) and (b), Before and after adsorption to Con A-Sepharose, using ^3H-glucosamine-Sindbis virus; (c) and (d), before and after adsorption to Con A-Sepharose, respectively, using ^3H-fucose-Sindbis virus. Symbols: (●), ^3H; (○), ^{14}C; V_o, void volume; V_t, total volume.*

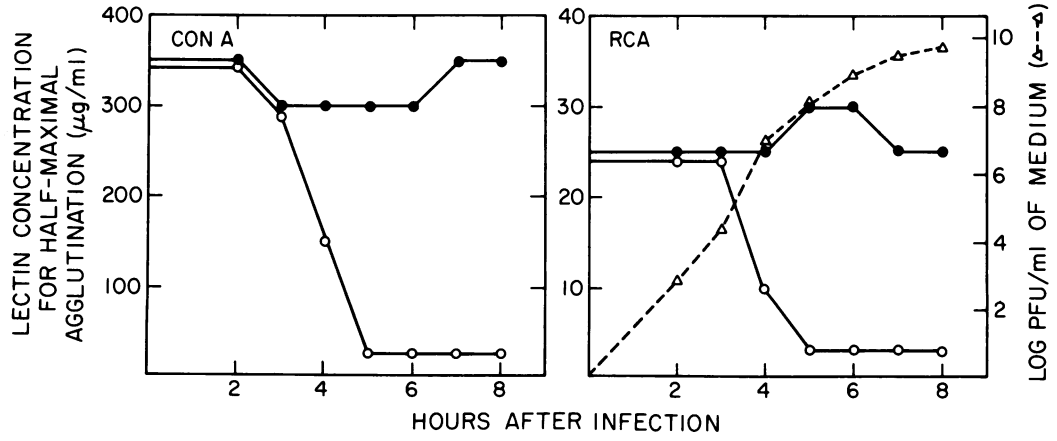

FIG. 2. *Agglutination of chick cells after infection by Sindbis virus. The growth curve applies to both Con A and RCA experiments. Symbols: (●), mock-infected; (○), infected.*

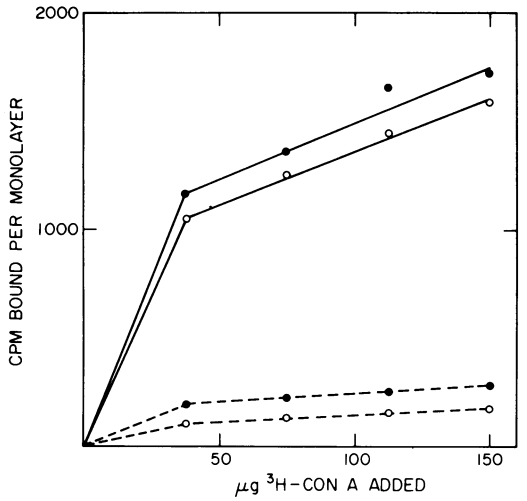

FIG. 3. *Binding of ³H-Con A to mock-infected chick cells and to cells infected by Sindbis virus. Symbols: (●), mock-infected; (○), infected; solid lines, no inhibitor; dotted lines, binding in the presence of 0.1 M α-methyl-D-mannoside. Procedures for infecting cells, preparation of ³H-Con A, and determination of ³H-Con A bound to monolayers have been described in Materials and Methods.*

modified to contain the virus-specific proteins (1, 22, 30). Thus, many new receptor sites for Con A and other lectins are inserted into the cell plasma membrane. It was interesting to find that the infected cells bound the same amount of Con A as did the uninfected cells. It is possible that the number of new binding sites inserted into the cell surface is small compared to the number of preexisting sites so that an increase after infection would not be seen. This seems unlikely, however, in view of the large amount of virus glycoprotein made after infection (up to 2% of the total cell protein late in the infection cycle is virus glycoprotein [29]) and in view of the large areas of budding seen in the electron microscope (6; Birdwell and Strauss, manuscript in preparation). It seems more likely that preexisting sites are lost during virus alteration of the cell surface. It is possible that part of the effect is due to steric hindrance among the Con A molecules; i.e. the Con A binding sites can be packed more tightly than can Con A molecules. The unit cell of crystalline Con A contains the equivalent of two Con A tetramers and has a volume of 5×10^{-19} cm³ (24). Thus, in the crystal, each Con A molecule requires the equivalent of 4×10^{-13} cm² in cross-sectional area, and the 5×10^{13} Con A molecules bound per petri plate would occupy at least 20 cm² of cell surface area. Since the area of the petri plate used is 22 cm², it is clear that a significant fraction of the cell surface area is occupied by Con A molecules. Furthermore, a budding virus particle represents a cluster of Con A binding sites, since there is room on the surface of a virion for several hundred Con A molecules. Thus, in those areas that bind Con A, the Con A molecules must be fairly tightly packed, and differences in the absolute number of potential binding sites available would thus be obscured.

Since infected cells bind the same amount of Con A as do the uninfected cells, the increased agglutinability of the infected cells must be due to changes in the topology of the cell surface (perhaps caused by budding virus) rather than to the insertion of the viral glycoproteins per se. In addition, this change occurs early in infection, after commencement of budding but when less than 1% of the final virus yield has been produced; virus-specific protein synthesis becomes detectable only after 3 h of infection (29). It is interesting to note that this stage of the Sindbis virus life cycle is characterized by rapid changes in two other cell surface properties as well; an increase in the ability of the infected cells to hemadsorb (7) and a decrease in the ability of infected cells to adsorb Sindbis virions under certain conditions (Pierce, Strauss, and Strauss, manuscript in preparation). An electron microscope study of these topological changes, using surface replica techniques, is in progress.

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