Location of Ferritin-Labeled Concanavalin A Binding to Influenza Virus and Tumor Cell Surfaces

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Concanavalin A (Con-A) was linked to ferritin with glutaraldehyde and chromatographed on Sepharose 6B to separate unconjugated Con-A and ferritin from covalently cross-linked molecules. Ehrlich ascites tumor cells were infected with WSA influenza virus, stained at intervals with the ferritin-labeled Con-A, and examined by electron microscopy. The surfaces of most mature viruses were specifically stained, providing direct evidence that influenza viruses maturing in this cell type have exposed Con-A receptor sites. The ferritin cores of the staining reagent were found at an average distance of 21.3 nm from the virus membrane and 10.8 nm from the uninfected cell membrane. This finding was interpreted to mean that the population of Con-A receptor sites on influenza virus particles is located at an average distance from the virus membrane twice that of the population of Con-A receptor sites found on uninfected cells. The structural elements of viral membranes can provide a reliable means for evaluating electron microscopy staining reagents, thereby enhancing their usefulness as probes for the study of membrane relationships.

Concanavalin A (Con-A), a phytoagglutinin derived from *Canavalia ensiformis*, binds specifically to several branched carbohydrates containing terminal nonreducing α -D-glucopyranosyl, α -D-mannopyranosyl, or α -D-fructofuranosyl residues (14, 15). It has been utilized extensively to monitor alterations in the surface membranes of cells transformed by various carcinogens (5). Many cell types, normally not agglutinable by Con-A, become so following tumor virus conversion (19, 20), but this may not be true of all oncogenic virus target cell systems (25). Furthermore, various cells infected with nononcogenic, enveloped ribonucleic acid (RNA) viruses have recently been shown to be agglutinable by Con-A, whereas uninfected cells are not (4, 36, 40). The recent finding that most normal and tumor virus-transformed cells have the same number of binding sites for Con-A (2, 8, 29) has led to a hypothesis that a rearrangement of, but not an increase in, Con-A sites may be responsible for the observed acquisition of cell agglutinability following virus infection (26). The possible role of the membrane components of enveloped viruses in the increased agglutinability of cells infected with both transforming and nontransforming viruses is unknown.

Recently, Calafat and Hageman attempted to show Con-A receptor sites on the surfaces of

mammary tumor viruses and Rauscher leukemia viruses by utilizing an indirect horseradish peroxidase labeling technique (6). They were able to demonstrate a thick layer of electron-dense, rather homogeneous precipitate along the membrane of both viruses. The coarse precipitates seen, however, did not permit precise localization of individual receptor sites on the spikes or "studs" of the virions (12). Since the molecular dimensions of ferritin (10), Con-A (17), and the surface projections of influenza virus (23, 32, 37, 38) are known, a ferritin-labeled concanavalin reagent was prepared and used to investigate the location of Con-A receptor sites on cell and influenza virus surfaces.

MATERIALS AND METHODS

Viruses. The WSA strain of neurotropic, tumor-adapted A₀ influenza virus was used throughout this study (24). It was grown in 10- to 11-day-old embryonated White Leghorn chicken eggs at 35 C for 48 hr. Both crude allantoic fluid and virus, concentrated 10 times in an ultracentrifuge, were stored at -70 C.

Buffers. Hemagglutination buffer (HAB; Difco, Detroit, Mich.), an isotonic sodium chloride solution buffered to pH 7.25 with Sorensen buffer, was used for manipulation of chemicals, viruses, and cells except where otherwise indicated.

Con-A. Three times crystallized Con-A (lot no. 20) supplied as a lyophilized powder by Miles Labora-

tories, Kankakee, Ill., was used for preparation of the fluorescein-conjugated and ferritin-labeled Con-A.

For use, the powder was dissolved in HAB, and the final concentration of Con-A was determined by its absorbance at 280 nm (1 mg/ml corresponds to 1.3 absorption units) (33).

Inhibitors of concanavalin-A. Methyl α -D-mannopyranoside (α -Memann) and sucrose competitively inhibit the Con-A receptor reaction, whereas galactose does not (27). α -Memann was obtained from Pfaltz Laboratories, Wankegan, Ill., and was made up as a 5% (w/v) solution in HAB. Sucrose and galactose were made up as 0.5 M stock solutions in HAB and diluted to a final concentration of either 0.1 or 0.2 M for use.

Hemagglutination tests. Hemagglutinin (HA) titers were determined utilizing 0.025-ml droppers and microdiluters in a microtiter system with "V" plates. A 0.5% suspension of chicken erythrocytes in HAB was utilized throughout. End points were determined and expressed as the reciprocal dilution.

Hemadsorption tests. The ability of virus-infected Ehrlich ascites tumor (EAT) cells to absorb erythrocytes was assessed using the procedure of Gerhard (13). Briefly, 0.9 ml of a tumor cell suspension was mixed with 0.1 ml of a 5% suspension of human type O erythrocytes or mouse erythrocytes and incubated for 1 hr at 4 C. The numbers of positive (two or more adherent erythrocytes) and negative cells were determined in a hemocytometer.

Preparation of fluorescein-conjugated Con-A. Con-A was labeled with fluorescein isothiocyanate (FITC) by mixing 0.15 mg of FITC/mg of Con-A in 0.1 M phosphate buffer at pH 7.4 for 18 hr (34). Free FITC was removed by passage of the mixture through a Sephadex G-25 column. The final concentrated stock solution had a 495 nm/280 nm optical density (OD) ratio of 1.1 and a protein concentration of 650 μ g/ml.

Equally brilliant staining of EAT cells occurred over a range of 13 to 100 μ g of FITC-Con-A/ 10^6 cells. This staining could be totally inhibited by addition of 0.1 M α -Memann or sucrose but not by addition of 0.1 M galactose. For routine staining of EAT cells, FITC-Con-A was used at a concentration of 20 μ g/ 10^6 cells.

Preparation of ferritin-conjugated Con-A. Ferritin-conjugated Con-A (Fer-Con-A) was prepared by a modification of a recently described glutaraldehyde-coupling method (27).

To a 0.5 M NaCl-0.05 M sodium phosphate buffer solution (pH 6.8) containing 216 mg of six times recrystallized horse spleen ferritin (Miles-Pentex) and 60 mg of three times crystallized Con-A, 0.5% glutaraldehyde (Ladd Research Industries, Burlington, Vt.) was added to a final concentration of 0.05%. The mixture was stirred for 1 hr at 23 C and then dialyzed against the same buffer containing 0.1 M NH_4Cl for 5 hr at 4 C to stop the reaction. The mixture was subsequently dialyzed against two changes of 100 volumes of the same buffer without the NH_4Cl for 24 hr. The preparation was centrifuged at 13,000 $\times$ g for 30 min to remove the slight amount of aggregated material. A 5.0-ml amount of the mixture was

applied to the bottom of an 88 by 2.5 cm upward-flow column packed with Sepharose 6B (Pharmacia Fine Chemicals, Piscataway, N.J.) and previously calibrated utilizing blue dextran, ferritin, Con-A, and cytochrome c molecular-weight markers as shown in Fig. 1. Five-milliliter fractions were collected and diluted 1:40, and their absorption values at 280 nm were recorded. Fractions were pooled into three groups as shown in Fig. 1. All three pools agglutinated EAT cells, whereas unconjugated ferritin did not. The protein concentration of each pool was as follows: I, 11.1 mg/ml; II, 3.5 mg/ml; and III, 6.7 mg/ml. The three pools were not concentrated further but were dialyzed exhaustively against HAB, filtered through a 450-nm membrane filter (Millipore Corp.), and stored at 4 C. Observation with an electron microscope revealed that pool I consisted primarily of large aggregates of ferritin (10 to 60 molecules/clump) and that it stained cells very poorly. Pool III contained singlets of ferritin and stained cells sparingly and erratically. Pool II, on the other hand, appeared to consist chiefly of ferritin singlets which stained cells strongly, reproducibly, and specifically as evidenced by the ability of α -Memann and sucrose to reduce or completely block the staining reaction. Pool II was used, at a final concentration of 2.8 mg/ml, as the Fer-Con-A staining reagent for all studies described.

EAT cells. Replication of WSA influenza A₀ virus in EAT cells in vivo has been previously described (24), and has been demonstrated by electron microscopy (3). This in vivo procedure was not utilized to minimize the possibility that Con-A receptor-rich substances present in the ascites fluid might adhere to the virus particles.

EAT cells were harvested from ICR mice 6 days after inoculation i.p. with 10^6 cells. The cells were washed four times in HAB after which 1.8×10^6 cells were resuspended in either 10 ml of HAB or 10 ml of HAB containing virus at 1.9×10^7 egg infectious dose (EID_{50})/ml. The cells and cell-virus mixtures were incubated for 20 min in an ice bath, washed two times with 10 volumes of HAB, resuspended in serum-free RPMI 1640 medium containing 100 units of penicillin and 100 μ g of streptomycin/ml, and *N*-2-hydroxyethylpiperazine-*N'*-2'-ethanesulfonic acid (Grand Island Bio-

FIG. 1. Chromatography of Fer-Con-A complexes on a calibrated Sepharose 6B column. Pool II was utilized as the staining reagent in this study (see Materials and Methods).

logical Co.). The cells at a final concentration of 3.6×10^6 /ml were incubated in Spinner flasks (Wheaton Celstir) at 35 C in a 5% CO₂-95% air atmosphere.

Fluorescent microscopy. EAT cells were washed two times at 4 C, resuspended in 0.2 ml of FITC-Con-A to yield a proportion of 10^6 cells/20 μ g of FITC-Con-A, and incubated at 4 C for 20 min. The cells were washed once and resuspended in a medium of 50% HAB-50% glycerin, and a drop of this suspension was mounted on a slide under a cover slip for viewing.

The stained preparations including those stained with α -Mennan-blocked FITC-Con-A were viewed with a Leitz microscope using an XBO 150-w light source and a BG-13 filter. Barrier eyepiece filters used were Kodak Wratten celluloid filters K2 and 57A. The K2 filter allows both red and green fluorescence to be seen, whereas the 57A allows only green to be seen.

Electron microscopy. To tubes in an ice bath containing 7.5×10^6 washed infected or uninfected EAT cells were added 0.2-ml volumes of cold staining reagents including unblocked pool II Fer-Con-A, Pool II Fer-Con-A in 0.1 M α -Mennan, sucrose or galactose, and unconjugated ferritin mixed with Con-A at a 3.6:1 (w/w) ratio. Cells were stained for 45 min at 4 C and then washed two times in 50 volumes of HAB. Cells were resuspended and fixed overnight in 0.5 ml of 2% glutaraldehyde-0.1 M cacodylate buffer, pH 7.4. They were then washed two times with cacodylate buffer and fixed in 1% osmium tetroxide for 1 hr at 23 C in the dark. Dehydration was accomplished by using two 10-min changes each of 50%, 70%, 95%, and absolute ethanol. The cells were then exposed to two 10-min changes of propylene oxide followed by 20-min exposures to each of three mixtures with polypropylene oxide: Epon 812 ratios of 4:1, 2:1, and 1:1. The cells were held overnight in the 1:1 mixture. Cell pellets were transferred to Beem capsules, centrifuged in a small amount of fresh Epon 812 to make a firm pellet, and were held at 23 C for 24 hr. Capsules were filled and placed in an oven at 40 C for 24 hr after which the temperature was increased to 60 C to complete the cure. Thin sections (25 nm) were cut on an LKB microtome using a diamond knife and were picked up on 100-mesh collodion carbon-coated grids. Sections were routinely stained with a saturated aqueous solution of uranyl acetate and lead citrate. Electron micrographs were taken using a Hitachi HU-11C microscope.

RESULTS

Agglutination of cells by Fer-Con-A. Fer-Con-A, at protein concentrations ranging from 0.28 to 2.8 mg/ml, agglutinated various cell types, indicating that this activity of Con-A had not been destroyed by the glutaraldehyde-coupling reaction. Figure 2 shows the appearance of one of these aggregates consisting of two Ehrlich ascites cells, a DBA/2 mouse erythrocyte and a DBA/2 mouse thymocyte. Evident along the margins of all cell membranes and between cells

at points where the membranes are in contact are numerous ferritin molecules.

In Fig. 2a and b, the Fer-Con-A appears to cross-link abutting cell membranes, and also stains cell membrane adjacent to the cross-linked regions, implying that the Fer-Con-A is not merely passively trapped between cells. Similar findings have been reported by others using mouse thymocyte suspensions (35).

Effect of temperature on the staining reaction. Infected and uninfected EAT cells, stained with Fer-Con-A at room temperature (23 C) for 30 min or more, consistently internalized the Fer-Con-A which had become bound to their outer membrane. Figures 3a and b illustrate this phenomenon in which the Fer-Con-A is clearly visible both at the surface of EAT cells and within vacuoles in the cytoplasm. The possibility cannot be excluded that some of these structures are merely cross-sections of invaginations of the cell membrane. However, whether invagination or vacuole, internalization of Fer-Con-A could be prevented by cooling all reagents and cells in an ice bath for 1 hr prior to and throughout the staining procedure. Figures 3c and d show that under these conditions Fer-Con-A could be restricted to the outer membrane of EAT cells.

Growth of WSA in EAT cells. Figure 4 presents the growth curve of WSA influenza virus in a typical EAT cell culture. Uninfected control cultures developed neither free HA nor hemadsorption. The viability of the cells in the uninfected culture was greater than 90% throughout the experiment, whereas the viability of the cells in the infected culture fell to 80% at 16.5 hr and 68% at 24 hr. Samples from both infected and uninfected cultures were prepared for electron microscopy at 16.5 and 20 hr.

The binding of Con-A to infected and uninfected cells was observed using FITC-Con-A. Figures 5b, c, and d show the typical patterns of staining seen in WSA-infected EAT cells 20 hr after incubation. Over 90% of the infected cells were quite brilliantly stained at this time. Patterns included fluorescence between agglutinated cells (5b), polar staining (5c), and full or partial ring fluorescence (5d). In marked contrast to this was the appearance of the uninfected cells at 20 hr as shown in Fig. 5a. Over 90% failed to show staining with FITC-Con-A at 20 hr. However, the majority of these uninfected cells proved to have receptor sites for Con-A when examined by electron microscopy following Fer-Con-A staining. The number of receptor sites as revealed by Fer-Con-A staining at 20 hr was markedly less on uninfected cultured EAT cells compared with WSA-infected EAT cells.

Staining of influenza viruses with Fer-Con-A. Figure 6 shows examples of the reaction of Fer-

FIG. 2. Agglutination of mouse cells by Fer-Con-A. Abbreviations: R, DBA/2 erythrocyte; E, Ehrlich ascites tumor cell; T, DBA/2 thymocyte. $\times 40,000$. Insets a and b, $\times 100,000$.

Con-A with WSA virus 20 hours after infection of EAT cells. It is clear in Figs. 6a and b that all viruses are stained at their surface with Fer-Con-A. Many ferritin markers are spaced along the virus

surfaces in a way that suggests interaction with the spikes of the viruses. In all experiments to date, over 90% of observed virus particles have stained positively with Fer-Con-A.

FIG. 3. Effect of temperature on Fer-Con-A localization during staining. *a* and *b*, Internalization of Fer-Con-A following staining at 23°C; *c* and *d*, restriction of Fer-Con-A to the cell surface by staining at 4°C. All, $\times 100,000$.

Figures 6c and e show virions which appear to be cross-linked or agglutinated by Fer-Con-A complexes. In Fig. 6d a single virion is clearly stained with Fer-Con-A. There is relatively uniform ferritin-membrane spacing, and again a clear suggestion that the Fer-Con-A is binding to the spikes of the virus.

Specificity of the reaction. All of the preceding reactions of Fer-Con-A with viruses and cells

could be almost totally blocked by including either 0.1 M sucrose or 0.1 M α -Memann in the staining reagent, but was not blocked by galactose (27). Figure 7a shows that Fer-Con-A fails to stain a normal EAT cell in the presence of 0.1 M sucrose. Figure 7b shows the inhibitory effect of 0.1 M α -Memann on Fer-Con-A staining of an infected EAT cell.

Ferritin-membrane spacing. By using a calibrated optical comparator (Brookstone Company, Peterborough, N.H.), the distances of individual ferritin markers from the virus or cell membrane were determined. Each measurement was made from the center of the ferritin molecule to the outer edge of the virus or cell membrane. Over 500 individual ferritin molecules were observed on stained virus particles and an equal number on normal uninfected EAT cells. Figure 8 shows the results of these measurements. Figure 8A shows the distribution of the distances of ferritin markers from the surface membrane of uninfected EAT cells. Most of the markers on EAT cells were found at distances ranging from 4 to 17 nm, whereas only 5% were present at distances of 18 to 25 nm. The mean distance of markers from the uninfected EAT cell membrane was 10.8 nm. In contrast to the uninfected EAT cell, Fig. 8B shows that the majority of markers

FIG. 4. Growth of WSA influenza virus in EAT cells in a Spinner culture at 35 C.

FIG. 5. Staining of EAT cells with FITC-Con-A after 20 hr of incubation at 35 C in a Spinner culture in serum-free RPMI 1640 medium. a, Uninfected control cells; b, c, and d, cells infected with WSA influenza virus.

FIG. 6. Staining of WSA influenza viruses with Fer-Con-A 20 hr after infection. *a* and *b*, $\times 80,000$; *c*, *d*, and *e*, $\times 200,000$.

on virus particles were found at distances ranging from 15 to 25 nm with a mean distance of 21.3 nm.

DISCUSSION

Various ferritin-labeled reagents have been employed to probe the surfaces of viruses and cells (1, 11, 30). A Fer-Con-A reagent similar to ours has been utilized to examine the surfaces of normal and transformed cells (26, 35), but previously has not been reported as used to stain viruses. In none of these studies were measurements made from ferritin cores to surface membranes.

The twofold difference in ferritin-to-membrane distances observed between cells and viruses (Fig. 8) most probably reflects intrinsic differences in the location of Con-A receptor sites on the two populations. Heterogeneity of the Fer-Con-A complexes might contribute to the intra-population variation observed, but should not cause a twofold difference between populations stained under identical conditions with the same preparation.

By utilizing the data available to us, as well as the known dimensions of influenza virus (23, 32, 37, 38), some of the characteristics of the Fer-Con-A complexes in our preparation can be

inferred. Since pool II Fer-Con-A agglutinates cells, there must be a minimum of two available Con-A receptor binding sites attached to each ferritin molecule. These could be on one or more multivalent Con-A molecules (39). Our Con-A chromatographed on Bio-Gel P-150 in the conjugation buffer at a molecular weight of $100,000 \pm 6,000$, indicating that it consisted of whole molecules (39). In addition, pool II is probably restricted to ferritin molecules with 1 to 5 attached Con-A molecules since the range of molecular sizes in the pool is approximately 6×10^5 to 10^6 daltons (Fig. 1).

The simplified models of Fer-Con-A illustrated in Fig. 9 could account for most of the observed ferritin core-to-membrane distances in Fig. 8. Assuming that the maximum length of the whole Con-A molecule is 8 nm, (17), that of glutaraldehyde is 1 nm, and that the radius of ferritin is 6 nm, (10), the maximum distance between the center of a ferritin molecule and the extreme end of an attached Con-A molecule would be 15 nm. If the length of the influenza virus spike is taken as 12 nm (23, 32, 38), then a fully extended Fer-Con-A complex with one Con-A molecule attached to ferritin could present its ferritin center

FIG. 7. Blocking of Fer-Con-A staining of EAT cells and influenza virus. (a) 0.1 M sucrose, $\times 40,000$. (b) 0.1 M α -Memmann, $\times 160,000$.

at a maximum distance of 27 nm from the virus membrane (Fig. 9, right side). Direct measurements (Fig. 8B) revealed a mean distance of 21.3 with less than 5% of ferritin molecules present at a 26- to 28-nm distance from the virus membrane. However, a 3- to 5-nm variation in intermolecular spacing between ferritin and concanavalin is possible depending on the binding configuration involved in the Fer-Con-A complex. This is appreciated by comparing the contracted model on the left side of Fig. 9 with the extended form on the right. The distances actually observed on viruses can be generated by utilizing a contracted model to position the ferritin core closer to the membrane.

Moreover, although an interspike distance of 5 to 10 nm (38) could theoretically accommodate an appropriately oriented Con-A molecule (Fig. 9, left side), most often the Con-A portion of the Fer-Con-A complex probably engages only those Con-A receptor sites near the ends of the virus spikes and not putative sites buried between adjacent spikes. Native Con-A, for example, with a maximum length of 8 nm readily agglutinates influenza virus particles (4, and P. A. Klein,

unpublished observations). A single Con-A molecule could not cross-link virus if available receptors were more than 8 nm down from the spike tip. Likewise, it is unlikely that Fer-Con-A complexes of the type shown in Fig. 9 are interacting with Con-A receptors located directly on the virus membrane. First, the overall length of these complexes is probably insufficient when considering the restriction imposed by the 12-nm diameter ferritin molecule at the top of the complex. Secondly, the virus membrane stripped of spikes has been shown to be devoid of Con-A receptor sites (21). Therefore, the complexes in our Fer-Con-A reagent need be no more elaborate than those depicted in Fig. 9 to explain the observed ferritin-membrane distances.

Finally, because 25% of the Con-A receptor sites on uninfected EAT cells are located at the same distances from the membrane (15 to 26 nm), as are 97% of the Con-A receptor sites on EAT-grown influenza virions, one might infer that the virus preferentially matures wherever these particular cellular glycoproteins are abundant, incorporating them into newly forming virus membrane. However, whereas the carbohydrate

FIG. 8. Distribution of the distances of ferritin markers from EAT cell membranes and WSA influenza virus membranes. (A) Distribution on uninfected EAT cells; (B) distribution on WSA influenza viruses; SEM, standard error of the mean; $\bar{X}$, mean; SD, standard deviation.

composition of influenza virus reflects that of the cell in which it was grown, the protein of the viral glycoprotein spikes (HA and neuraminidase) is determined by the viral genome (9, 18, 31). Thus, it is very unlikely that Con-A receptor sites on the virus are situated on preexisting cellular glycoproteins that have been simply incorporated by the virus during its maturation. More likely, those sugars which constitute Con-A receptors are deposited onto influenza virus-specific proteins by host cell glycosyl transferases. The demonstration that host cell sialyl transferase can transfer sialic acid to Sindbis virus glycoprotein supports this notion (16). The hemagglutinin glycoprotein of the BEL strain of influenza A₀ virus has two "heavy" chains each containing approximately 15% carbohydrate (7.6% glucosamine, 1.1% fucose, 4.8% mannose, and 1.5% galactose) and two "light" chains each containing approximately 1.5% glucosamine (22). The proposition that the mannose-like Con-A receptor sites are associated with the carbohydrate on the "heavy" polypeptide chains of the influenza HA could be readily tested using this strain.

These studies and those of others with different myxoviruses (4), paramyxoviruses (4), rhabdoviruses (4), leukemia viruses (6, P. B. Dent, Fed. Proc. 31:805, 1972), arboviruses (28), mammary tumor viruses (6), and Australia antigen (hepatitis virus) (7), support the notion that the mannose-like receptor for Con-A is an obligate structural component of many diverse animal viruses. Furthermore, our studies have demonstrated the potential value of employing known virus membrane dimensions to assess the characteristics of ferritin conjugates to be used as probes of unknown biomembrane surfaces.

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FIG. 9. Interaction of Fer-Con-A complexes with the glycoprotein spikes (hemagglutinin illustrated) of WSA influenza virus. Each complex consists of 1 ferritin and 1 Con-A molecule linked through 1 or more glutaraldehyde molecules. Complex on the left is shown binding to hypothetical Con-A receptors between adjacent spikes. Complex on the right is binding to Con-A receptors on the tip of a spike. Two alternative Fer-Con-A configurations are shown.

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