Ferritin-Conjugated Plant Agglutinins as Specific Saccharide Stains for Electron Microscopy: Application to Saccharides Bound to Cell Membranes

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ABSTRACT An electron microscopic stain for specific saccharides was prepared by the conjugation of ferritin to concanavalin A, a plant agglutinin that specifically binds to oligosaccharides containing terminal d-glucose, d-mannose, or sterically related sugar residues. A technique was developed to allow topological visualization of erythrocyte and other membranes by means of transmission electron microscopy, and the distribution of the binding sites for ferritin-concanavalin A on such membrane preparations was determined. The conjugate was found to bind specifically to the outer, but not the inner, surface of erythrocyte membranes. The number of conjugate molecules bound per unit area of the membrane was larger for rabbit than for human erythrocytes.

Complex oligo- and polysaccharides are known to be associated with the plasma membranes of a wide variety of cell types (1, 2). Electron microscopic studies of membrane-bound polysaccharides have utilized staining techniques adapted from light microscopy, such as colloidal-iron and colloidal-thorium staining (3–7) for acid mucopolysaccharides, or the periodic acid–silver methenamine procedure (8–13) for 1,2-dihydroxy alcohols and α-amino alcohols. These procedures suffer from limitations of specificity and of inadequate resolution.

Plant agglutinins are commonly occurring proteins (14) that bind to specific sugar ligands; several of these proteins have been isolated and characterized. One of these is concanavalin A, an agglutinin isolated from Jack beans (15), which binds reversibly to oligosaccharides containing terminal α-D-glucopyranosyl, α-D-mannopyranosyl, and sterically related sugar residues (16, 17), but not to other oligosaccharides. Concanavalin A has recently been used for gross studies of the surface architecture of cell membranes (18–20). We report the preparation of a specific electron microscopic stain for such terminal sugar residues by the conjugation of concanavalin A to ferritin (21), and the use of this conjugate to determine the distribution of concanavalin A binding sites on erythrocyte membranes. We have also developed a method for preparing membrane specimens to allow observations of the topological distribution of ferritin conjugates on membrane surfaces.

MATERIALS AND METHODS

Horse-spleen ferritin (6× recrystallized, Miles-Pentex) was further purified by crystallization and ultracentrifugation (22). Concanavalin A (Miles-Yeda) was obtained as a pure protein by elution from an adsorbent column (16). Glutaraldehyde was freshly prepared by distillation of 50% glutaraldehyde obtained from Union Carbide.

Ferritin-conjugated concanavalin A (Fer-Con A) was prepared by the glutaraldehyde-coupling method of Avrameas (23), since alkaline pH values had to be avoided to prevent aggregation of concanavalin A (24). To a solution containing 9% ferritin and 2.5% concanavalin A in a buffer of 0.5 M NaCl-0.05 M sodium phosphate (pH 6.8) was carefully added, with stirring, freshly distilled 0.5% glutaraldehyde, to a final concentration of 0.05%. After 45–60 min at room temperature, the mixture was dialyzed against the same buffer containing 0.1 M NH₄Cl for 3–4 hr at 4°C, and then dialyzed exhaustively against buffer. Any large aggregates were removed by centrifugation at 10,000 × g for 30–45 min. The preparation of ferritin-conjugated rabbit anti-(human) spectrin antibodies (Fer-antibody) has been described elsewhere (22, 25).

Fer-Con A and the ferritin-antibody conjugates were separated from unconjugated proteins by chromatography on columns of Biorad Agarose A 1.5 (22), and were concentrated to 2–5 mg protein per ml (in the pH 6.8 buffer for Fer-Con A, and 0.05 M sodium phosphate, pH 7.5, for the ferritin-antibodies) before use.

Freshly drawn human (B+) and rabbit erythrocytes were washed by several centrifugations in 0.9% saline, and were finally suspended at a concentration of 50%. A small volume of this solution was dropped onto a surface of distilled water. Most of the cells entered the water, but a small fraction of the cells apparently lysed at the air–water interface and surface tension caused their membranes to spread flat. Such mem-

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Abbreviations: Fer-Con A, ferritin-conjugated concanavalin A; Fer-antibody, ferritin-conjugated rabbit anti-(human) spectrin antibodies.
Fig. 2. A mounted rabbit erythrocyte ghost stained with a solution of ferritin-conjugated concanavalin A containing 100 mM D-galactose. The inner (i) and outer (o) surfaces of the membrane are distinguishable by their absorbances in the micrograph. The insert shows the entire ghost, and the brackets indicate the area magnified in the figure. Bar equals 0.2 μm; insert bar equals 1 μm.

Fig. 3. Same as Fig. 2, except that the staining solution of ferritin-conjugated concanavalin A contained 100 mM sucrose, an inhibitor of concanavalin A.

Fig. 4. Same as Fig. 2, except that the mounted rabbit erythrocyte ghost was stained with ferritin-conjugated anti-(human) spectrin.
branes were picked up on carbon-coated collodion films on electron microscope grids when the grids were lowered onto the interface. The grids were then conditioned with a solution of bovine serum albumin (26) and, without drying, a large drop of the Fer-Con A or ferritin-antibody solution was applied to each of the grids. After 3–5 min incubation at room temperature, the grids were washed by floating them face down on 6–10 consecutive fresh buffer surfaces and, finally, on distilled water. No further staining was done. After air-drying, the specimens were examined in a Phillips model 300 electron microscope.

**RESULTS**

Erythrocyte membranes prepared as described were often intact, double-membrane-thick ghosts spread completely flat on the carbon–collodion coated grid for the electron microscope. Sometimes, however, a whole ghost became attached to the supporting film so that its upper membrane had broken and folded back, revealing the inner surface of the lower membrane (Fig. 1 and inserts to Figs. 2–4). The outer and inner membrane surfaces on the same ghost were easily differentiated by their absorbancies on the micrograph, since the outer surface was exposed above a double thickness of membrane and the inner above a single thickness (see Fig. 1). If such rabbit or human ghosts were treated with Fer-Con A as described, only the outer surfaces of the membranes were labeled with ferritin (Fig. 2). This labeling was specific: the addition of 100 mM sucrose (an inhibitor of Con A attachment) to the Fer-Con A solution prevented labeling (Fig. 3), whereas 100 mM D-galactose (not an inhibitor) had no effect (Fig. 2).

The protein spectrin has been shown to be localized on the inner surface of the human erythrocyte membrane by the use of ferritin-conjugates of rabbit-anti-(human) spectrin antibodies (22). Human and rabbit spectrin are cross-reactive antigens (29). Therefore, ferritin–anti-spectrin can be used as a specific stain for the inner surfaces of both human and rabbit erythrocyte membranes. As expected, the surfaces termed "inner" in Figs. 2 and 3 were exclusively ferritin-labeled with Fer-antibody (Fig. 4). The possibility that spectrin might simply obstruct the binding of Fer-Con A to the inner-membrane surface of the erythrocyte was eliminated as follows. The spectrin was removed from mounted broken ghosts by treatment with a buffer containing 0.02% EDTA and 5 mM β-mercaptoethanol (27, 28) for 15 min at room temperature. Such treated ghosts no longer bound Fer-antibody to their inner surfaces. The distribution of Fer-Con A, however, was the same as in Fig. 2. We conclude that the concanavalin A binding sites are exclusively on the outer surfaces of the human and rabbit erythrocyte membranes.

In parallel experiments, the relative amount of binding of Fer-Con A to the outer-membrane surfaces of human (Fig. 5) and rabbit (Fig. 6) erythrocytes was compared. No attempt was made to saturate the binding sites, but substantially more binding was observed with the rabbit erythrocyte membranes. In addition, the ferritin molecules appeared to be in larger clusters on the rabbit than on the human membranes. These observations provide the ultrastructural correlate of the finding (30) that intact rabbit erythrocytes are agglutinated by much lower concentrations of concanavalin A than are human erythrocytes.

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**Fig. 5.** An area of a mounted human erythrocyte ghost stained with ferritin-conjugated concanavalin A. Bar equals 0.2 μm.

**Fig. 6.** Same as Fig. 5, except that a rabbit erythrocyte ghost was stained with ferritin-conjugated concanavalin A in a parallel experiment.
DISCUSSION

These experiments demonstrate that ferritin conjugates of plant agglutinins can be used as electron microscopic stains for specific sugar residues in a manner similar to ferritin–antibody conjugates. In addition to concanavalin A, we have also successfully prepared and applied conjugates of ricin with ferritin. Ricin has a different binding specificity than concanavalin A; it is specific for terminal D-galactose, L-rhamnose, and sterically similar sugar residues (31). Many other suitable plant agglutinins are known, with distinctive saccharide-binding specificities (14). A battery of reagents for the electron microscopic localization of various oligosaccharides in cellular preparations is thus potentially available. New embedding techniques that have been developed (32) may prove to be useful for intracellular staining with ferritin–plant agglutinin conjugates.

Experiments with Fer–Con A and erythrocyte membranes have strikingly revealed the asymmetry of concanavalin A binding sites on the two surfaces of the membrane. That this absolute asymmetry is also observed with ferritin–ricin conjugates, and with membranes of various cell types besides erythrocytes, will be demonstrated elsewhere (G. L. Nicolson and S. J. Singer, in preparation). The essentially exclusive localization of sialic acid residues to the outer surfaces of erythrocyte (33) and of liver cell plasma membranes (7) has already been shown. These results have, therefore, led us to conclude that this asymmetry is a general phenomenon for the oligosaccharides of plasma membranes of eucaryotic cells, and to suggest that it is the result of a specific mechanism for membrane biogenesis (G. L. Nicolson and S. J. Singer, in preparation).

Preparation of erythrocyte membranes by lysis at an air-water interface for studies of the two-dimensional distribution of ferritin–plant agglutinin and ferritin–antibody conjugates on membrane surfaces has proved to be generally useful. We have employed it to determine the distribution of Rh-antigenic sites on human erythrocyte membranes (G. L. Nicolson, S. P. Masouredes, and S. J. Singer, in preparation) and, with slight modification, allelic forms of the H-2 histocompatibility antigens on several types of mouse cells (G. L. Nicolson, R. Hyman, and S. J. Singer, in preparation). Experiments are also in progress to study the distribution of concanavalin A and other plant agglutinin binding sites on the surfaces of normal and oncogenic virus-transformed cells (18–20).

As this paper was being written, the preparation of a fluorescein conjugate of concanavalin A, and its use for the light-microscopic analysis of concanavalin A binding to cells during various parts of their cell cycles, was described (34).

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