LOCALIZATION OF INTESTINAL SUCRASE-ISOMALTASE COMPLEX ON THE MICROVILLOUS MEMBRANE BY ELECTRON MICROSCOPY USING NONLABELED ANTIBODIES

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ABSTRACT

Microvillous vesicles isolated from rabbit small intestine showed a trilaminar membrane with a rather smooth surface, which was apparently not affected by papain solubilizing sucrase-isomaltase complex or by trypsin unable to solubilize it. When microvillous vesicles or trypsinized ones were incubated with immunoglobulin G against the sucrase-isomaltase complex or monovalent fragments therefrom, an apparently continuous electron-opaque layer ~180 Å in width appeared around the external surface of vesicles. Such a layer was not formed on papainized vesicles. Microvillous vesicles and trypsinized ones negatively stained with phosphotungstate showed a great number of particles protruding ~150 Å from the membrane surface, but papainized vesicles did not. The particles existed close to one another and appeared to form a particulate layer 150 Å in width on the surface. The antibodies, whether they were divalent or monovalent, increased the width of the layer to ~200 Å and obscured the fine particulate structure of intact and trypsinized vesicles. Papainized vesicles retained their smooth surface upon interaction with the antibodies. These results, together with those with the Triton-solubilized sucrase-isomaltase complex (Nishi and Takesue, 1978, *J. Ultrastruct. Res.*, 62:1–12), indicate not only that sucrase-isomaltase complexes are located close to one another on the membrane, but also that they or at least their protein portions protrude ~150 Å from the surface of the trilaminar membrane.

KEY WORDS sucrase-isomaltase complex · rabbit small intestine · microvillous vesicles · membrane surface · immuno-electron microscopy · ruthenium red staining

The intestinal sucrase-isomaltase complex (SI complex) probably bears a close relationship to the transport of some sugars across the microvillous membrane (15, 23, 27, 31). In this respect, its exact location on the microvillous membrane has been a subject of great interest. Negatively stained microvillous membranes are studded with a great number of doughnut-shaped (110 Å in outer diameter) or 50–60-Å monomeric particles, at least some of which have been implicated in the SI complex (8, 19, 33). Benson et al. (2) have cast suspicion on this proposed relationship. Although the entity of the above-mentioned particles is still under controversy, the fact that the SI complex is exposed on the outer surface of the membrane has been established (for review, see reference 27).

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The SI complex has been solubilized with Triton X-100 (Rohn & Haas Co., Philadelphia, Pa.) and purified to a homogeneous state (28, 36). Like other membrane surface proteins such as erythrocyte glycophorin (26), microsomal cytochrome b_5 (29) and cytochrome b_5 reductase (30), and brush border aminopeptidase (18), the SI complex is considered to be composed of a large hydrophilic, enzymatically-active glycoprotein portion and a much smaller hydrophobic portion which is supposed to anchor the whole molecule to the membrane (28, 36). This suggests that the SI complex emerges almost entirely from the outer surface of the membrane, but how far it protrudes from the surface remains a question. In a previous paper (21), we have reported that Triton-solubilized SI complex has the dimeric structure in which two subunits similar in shape and size (~45 × 65 Å) are united to each other at their long axes, and that it has a hydrophobic portion at or near one end of its elongated molecule, suggesting that the SI complex does not "lie" on the membrane surface but "projects" from the surface. On the other hand, Gitzelmann et al. (4, 5), on the basis of results obtained by electron microscopy and the use of ferritin-labeled antibodies, have suggested that the SI complex antigen is located at the membrane surface and does not project into the intermicrovillous space.

In the ferritin-labeled antibody technique, the marker we can observe is usually ferritin cores, and neither antibodies nor antigens are visualized. If an antigen is present at a high density in the region in question, it may be possible that the antigen-directed antibodies themselves or the antigen-antibody complex give an electron opacity sufficient to be detected upon their interaction (1, 22). The intestinal SI complex probably satisfies the above-mentioned condition, because it is estimated, from the specific activities of the purified and the membrane-bound enzymes (33, 35), to amount to >10% of the total protein of microvillous membranes. Furthermore, about four molecules of antibodies against the SI molecules can be bound to different antigenic sites widely distributed over the SI complex molecule at the equivalence zone (38, 21). Therefore, we have attempted to localize the SI complex on the microvillous membrane by electron microscopy and the use of nonlabeled antibodies. The results obtained demonstrated that the complex particles protrude ~150 Å, close to one another, from the outer surface of the trilaminar membrane structure. Some of this work has been presented in summary form (20).

MATERIALS AND METHODS

Materials

Fragmented microvilli or microvillous vesicles of rabbit small intestines were purified from the microsomal fraction by centrifugation on a sucrose density gradient (32, 33) or, in some cases, from isolated brush-border sheets (39). No significant differences were detected in the enzymatic activities (the specific activity of sucrase was at least 10 times higher in isolated microvillous vesicles than in homogenate), electron microscopic images, and agglutinability by antibodies between the preparations of microvillous vesicles obtained by the two methods.

Anti-SI complex immunoglobulin G (anti-SI IgG) was isolated from the antisera raised in a goat against rabbit-intestinal papain-solubilized SI complex, as described previously (40). Monovalent fragment (anti-SI Fab) was obtained by treatment of anti-SI IgG with papain, followed by gel filtration on Sephadex G-75 (37). Control gamma globulin was isolated by ammonium sulfate fractionation from the sera of the pre-immunized goat. Anti-Si IgG is not only specific for the SI complex (36, 37, 40) but also interacts with both subunits, sucrase and isomaltase, of the complex (38). Anti-SI Fab is also bound to antigenic sites widely distributed over the SI complex (21).

Analysis and Assays

Protein was determined by the method of Lowry et al. (11), using bovine serum albumin as the standard. Sucrase, trehalase and leucine β-naphthylamidase were assayed as described previously (37).

Treatment of Microvillous Vesicles with Trypsin or Papain

Microvillous vesicles (5 mg of protein/ml) were treated with 0.1% trypsin or papain (Boehringer Mannheim Biochemicals, Indianapolis, Ind. at 37°C for 60 min and then centrifuged at 78,000 *g* for 60 min, as described previously (34). The precipitate was suspended in phosphate-buffered saline and used as trypsinized or papainized vesicles. In general, sucrase was solubilized >80% by papain, but <15% by trypsin.

Incubation of Microvillous Vesicles with Antibodies

Different preparations of microvillous vesicles were incubated with a little excess of anti-SI IgG or Fab for 60 min at 37°C and then for 20 h at 4°C, as described previously (34). Agglutinated vesicles were collected by centrifugation at 1,000 *g* for 30 min, and submitted to electron microscopic examination. When either papain-

ized vesicles or anti-SI Fab were used as a reactant, no significant agglutination occurred. In those cases, therefore, incubated vesicles were collected by ultracentrifugation. The degree of vesicle agglutination by antibodies was calculated by assaying the sucrase, trehalase, and leucin β-naphthylamidase activity as described previously (37).

Staining of Microvillous Vesicles with Ruthenium Red

To stain microvillous vesicles with ruthenium red, they were precipitated by ultracentrifugation and divided into two portions. One portion of the pellet was stained with ruthenium red according to Luft (14), and the other as control was also processed in the same way except that the fixative buffers used did not contain the dye. Stained and control fixed samples were submitted to the procedure for electron microscopy described below.

Processing for Electron Microscopy

Treated microvillous vesicles collected by centrifugation were divided into two portions, one for thin sectioning and the other for negative staining. For thin sectioning, vesicles in the pellet were fixed for 60 min at 4°C in 2.5% glutaraldehyde-2% paraformaldehyde in 0.05 M phosphate (pH 7.4), postfixed for 2 h at 4°C in 1% osmium tetroxide (pH 7.4), dehydrated in graded ethanol series, and embedded in Epon 812 (12). Sections ~600 Å thick were cut with an ultramicrotome (LKB Instruments, Inc., Rockville, Md.; Ultrotome type 4801-A). The sections were doubly stained with aqueous uranyl acetate and lead citrate (24, 25). For negative staining, precipitated vesicles were suspended in 0.4% sucrose at a protein concentration suitable for electron microscopic examination, and processed as described previously (21). The specimens were examined in an electron microscope (Hitachi HU-11B), equipped with an anti-contamination device, operating at 75 kV, and at a direct magnification of 50,000. The electron microscope magnification was calibrated with a germanium-shadowed carbon replica of a ruled diffraction grating. The width of the electron-opaque layer from the outer membrane surface in section was measured on the region of vesicles where the trilaminar membrane structure was clearly made visible. The protrusion of the particulate layer from the membrane surface in negative staining was measured on the edge of vesicles. These measurements were performed on prints magnified three times with a scale magnifier of 0.1 mm subdivision. The mean width of the electron opaque layer and the particulate layer was calculated for each group with at least 42 measurements.

RESULTS

Thin-Sectioned Microvillous Vesicles

Almost all the fragmented microvilli used in this study were closed vesicles which were enclosed by a trilaminar membrane of a rather uniform thickness (~100 Å) (Fig. 1*a*). The external surface of the membrane was very poorly overlaid with fuzzy structures even though osmium tetroxide-fixed sections had been subjected to a double staining with uranyl acetate and lead, which has been reported to be most effective in demonstrating the fuzzy coat (7). Microvillous vesicles were more or less filled with filamentous material which presumably originated from the core filaments of microvilli. As reported previously (34), some hydrolases bound to the microvillous membrane, e.g., sucrase and leucine β-naphthylamidase, were quantitatively solubilized by papain, but not by trypsin, whereas the trilaminar structure of the membrane was not apparently disintegrated either by papain or by trypsin (Fig. 2*a* and *b*).

Thin-Sectioned Microvillous Vesicles Agglutinated by Anti-SI IgG

Anti-Si IgG agglutinates microvillous vesicles, and the agglutinated vesicles are precipitated by low-speed centrifugation (36). When microvillous vesicles agglutinated by anti-SI IgG were examined by electron microscopy, a new layer of high electron opacity was found to cover the external surface of the membrane and to surround the whole surface of each vesicle (Fig. 1*b*). It was apparently of a uniform thickness, and was estimated to be 181 (± 24) Å wide at the region of the unit membrane cut normal to the plane of section. Like intact microvillous vesicles, trypsinized vesicles were agglutinated by anti-SI IgG, and the agglutinated vesicles showed a very similar electron-opaque layer on their external surface; its width was 178 (± 26) Å (Fig. 2*c*). On the other hand, papainized vesicles were scarcely agglutinated by anti-SI IgG, and no electron-opaque layer was seen on the surface of the vesicles collected by ultracentrifugation after incubation with the antibody (Fig. 2*d*). Sometimes, fuzzy structures were observed locally on the outer surface of some papainized vesicles incubated with antibodies; possibly, this may be related to the SI complex remaining bound to the vesicles.

Control gamma globulin did not agglutinate either intact or trypsinized vesicles, and did not form any electron-opaque structure on the membrane surface.

Negatively Stained Microvillous Vesicles

The surface of microvillous vesicles was studded with a great number of particles when viewed as

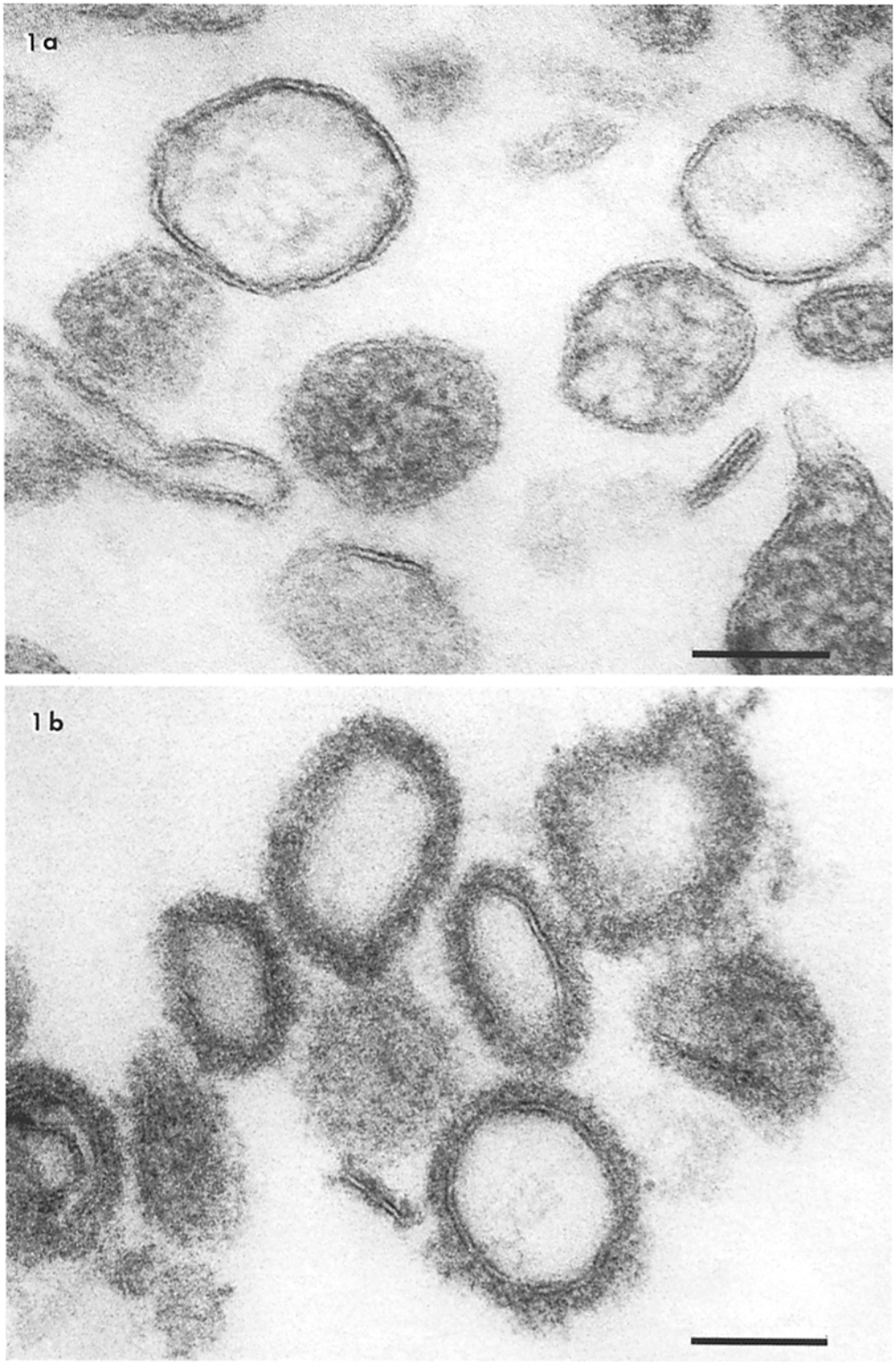

FIGURE 1 (*a*) Purified microvillous vesicles fragmented by sonication. The vesicles show a rather smooth surface and appear to be more or less filled with filamentous material. (*b*) Microvillous vesicles agglutinated with anti-SI IgG. A new electron-opaque layer of rather uniform thickness was seen surrounding the whole external surface of each of microvillous vesicles. No other significant differences in morphology were observed between Fig. 1 *a* and *b*. Bars, 0.1 μm. × 200,000.

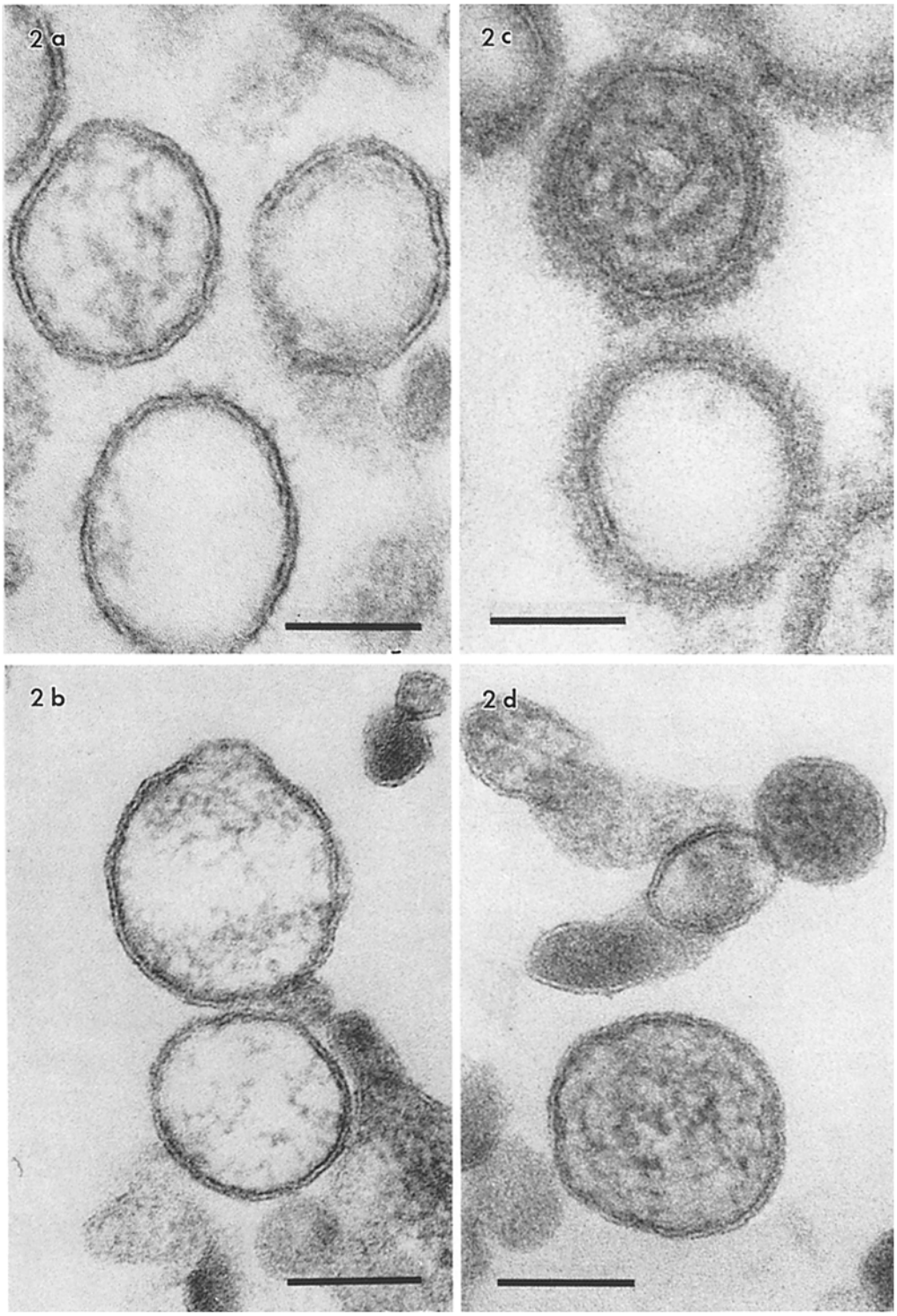

FIGURE 2 (*a*) Microvillous vesicles treated by 0.1% trypsin for 60 min at 37°C. No significant changes in morphology of the vesicles were induced by the trypsinization. (*b*) Microvillous vesicles treated by 0.1% papain for 60 min at 37°C. ~85% of the sucrase was solubilized by the treatment, but the width of the trilaminar membrane was seemingly unchanged. (*c*) Trypsinized microvillous vesicles shown in Fig. 2*a* were incubated with anti-SI IgG, and agglutinated vesicles were examined in thin sections. The external surface of the agglutinated vesicles was covered with a new electron-opaque layer very similar to that seen with control vesicles agglutinated by anti-SI IgG (Fig. 1*b*). (*d*) Papainized microvillous vesicles incubated with anti-SI IgG. The vesicles were not agglutinated by the antibody, so they were collected by ultracentrifugation after the incubation. Only very local fuzzy structures were seen on the external surface of the vesicles. Bars, 0.1 μm. × 200,000.

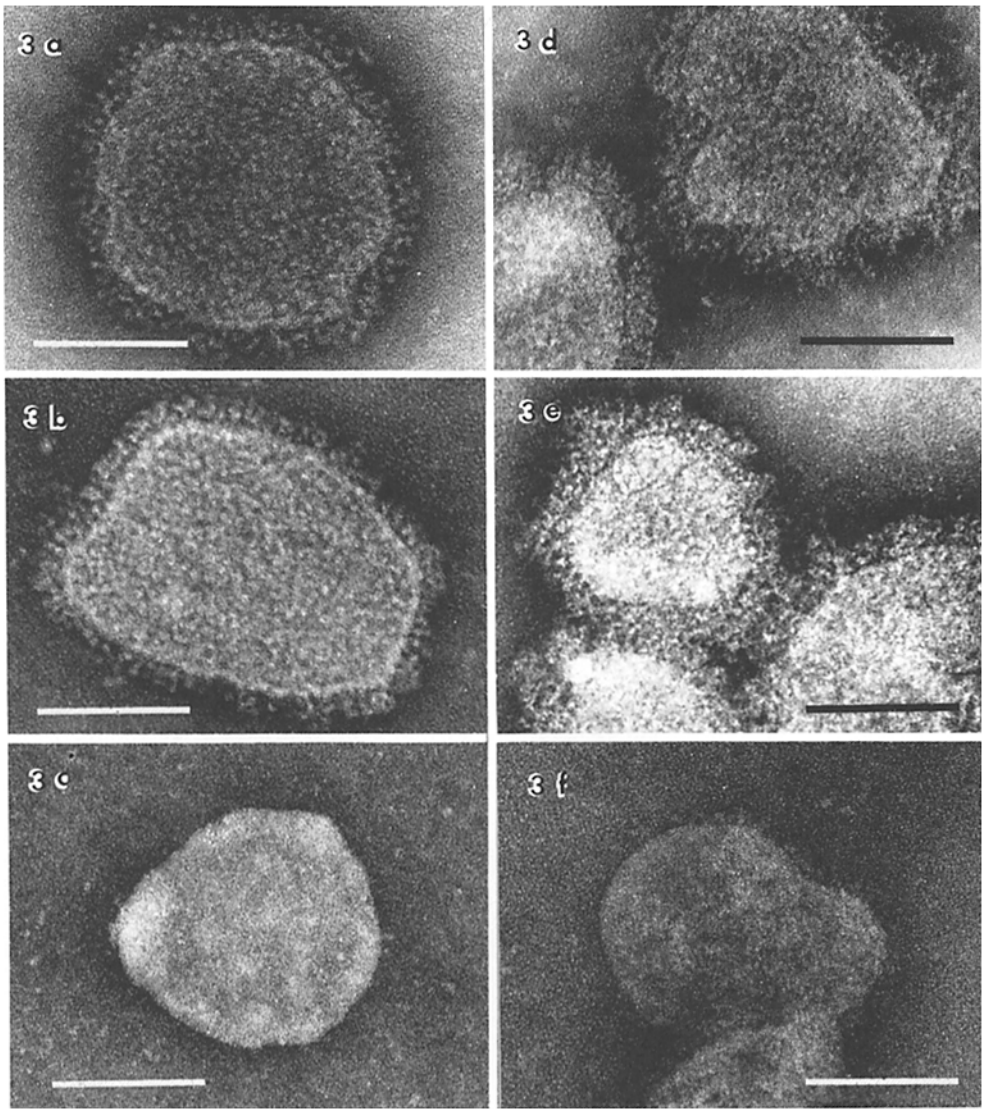

FIGURE 3 Electron micrographs of negatively stained microvillous vesicles exposed to different treatments. (*a*) Nontreated microvillous vesicles. (*b*) Trypsinized microvillous vesicles of the same preparation as shown in Fig. 2*a*. Microvillous vesicles shown in Fig. 3*a* and *b* are the same in appearance; the surface of the membrane is studded with a great number of particles, and the edge of the vesicles seems covered with a particulate layer ~150 Å wide. (*c*) Papainized microvillous vesicles of the same preparation as shown in Fig. 2*b*. The particles have been removed almost completely from the surface of the membrane, and the particulate layer covering the edge is not observed at all. (*d*) Nontreated microvillous vesicles agglutinated by anti-SI IgG. The fine particulate structure of the membrane surface and the peripheral layer are greatly obscured, and the width of the layer is increased to ~200 Å. (*e*) Trypsinized microvillous vesicles agglutinated by anti-SI IgG. The same morphological changes as seen in Fig. 3*d* are also noted here. (*f*) Papainized microvillous vesicles incubated with anti-SI IgG. Some particle-like structures are seen locally on the edge of the vesicles. Bars, 0.1 μm. × 200,000.

negatively stained preparations, and it appeared as if a particulate layer 151 (± 17) Å wide covered the whole surface of each vesicle (Fig. 3*a*). As reported previously (19), these particles were removed from the surface by papain (Fig. 3*c*) but not by trypsin (Fig. 3*b*). A few particles remained

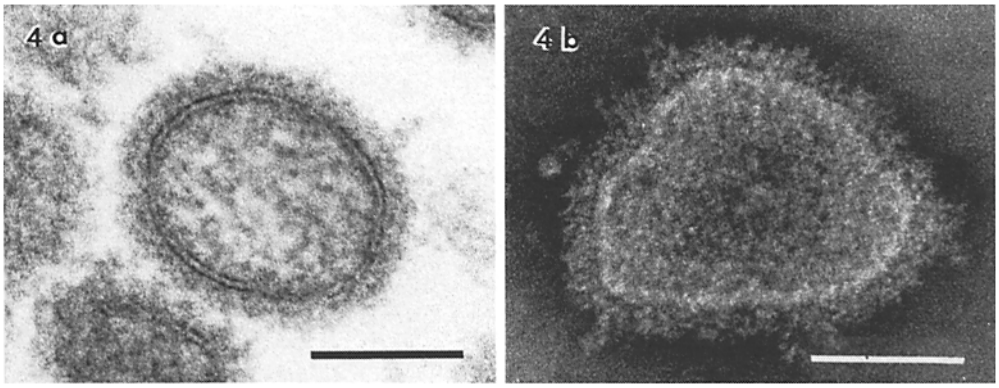

FIGURE 4 Microvillous vesicles incubated with anti-Fab. In this case, incubated vesicles were not agglutinated by anti-SI Fab, so the vesicles were collected by ultracentrifugation. (*a*) Thin section. The same electron-opaque layer as seen in Fig. 1*b* is observed on the external surface of the vesicle. (*b*) Negative staining. The same morphological changes as given by anti-SI IgG have been induced by anti-SI Fab. Bars, 0.1 μm. × 200,000.

bound to the surface of some papainized vesicles, which is probably related to the fact that in this case ~15% of the original sucrase activity remained still associated with the vesicles after papainization.

Negatively Stained Microvillous Vesicles Agglutinated by Anti-SI IgG

When microvillous vesicles agglutinated by anti-SI IgG were negatively stained with phosphotungstate, the width of the particulate layer seen on the edge of the vesicles increased from 150 Å to ~200 Å all around the edge, and its fine particulate structure was greatly obscured over the whole surface (Fig. 3*d*). The same result was obtained with trypsinized vesicles (Fig. 3*e*). On the other hand, the surface of papainized vesicles remained almost unchanged when they were incubated with anti-SI IgG, though local irregularities were sometimes seen on the edge of a few vesicles (Fig. 3*f*).

Microvillous Vesicles Incubated with Monovalent Antibodies

Monovalent antibody toward SI complex, anti-SI Fab, did not induce the agglutination of microvillous vesicles but it effected changes, quite similar to those induced by an anti-SI IgG, in the morphology of the membrane surface of microvillous vesicles (Fig. 4). In thin sections, the Fab formed a new electron-opaque layer 177 (± 20) Å wide on the whole external surface of the vesicles (Fig. 4*a*). In negatively stained vesicles, the fine structure of the particulate layer covering the vesicle surface was greatly obscured, and the width of the layer was extended from 150 to 200 Å (Fig. 4*b*).

Microvillous Vesicles Stained with Ruthenium Red

When intact and trypsinized microvillous vesicles were stained with ruthenium red, a substance which stains primarily acidic mucopolysaccharides (13, 14), an extremely electron-opaque layer 140–150 Å wide appeared on the whole external side of the trilaminar membrane (Fig. 5*a*). Except for some fuzzy structures, no such layer was seen on the papainized vesicles stained with the dye (Fig. 5*b*). The innter surface of the trilaminar membrane remained entirely unstained in all vesicle preparations.

DISCUSSION

Fragmented microvilli used in this work were closed vesicles limited by a trilaminar membrane with a rather smooth surface. The fuzzy coat associated with the lumenal surface of the epithelial cell seems to have been removed from the membrane surface during isolation of microvillous vesicles. However, the results with ruthenium red staining indicate that some polysaccharide-containing material is present in a layer 140–150 Å thick on the whole external surface of the vesicles, though it cannot be visualized by the staining of osmium-tetroxide-fixed sections with uranyl acetate and lead, which has been reported to be most

effective in demonstrating the glycocalyx (7). The ruthenium-red-positive layer is approximately the same in width as the particulate layer seen on the edge of the negatively stained vesicles. Like the SI complex and aminopeptidase (34), both layers can be removed from the membrane surface by papain but not by trypsin. When the particulate layer seen on negatively stained vesicles is widened to 200 Å by anti-SI IgG, a new electron-opaque layer ~180 Å in width appears around the outer surface of the thin-sectioned membrane which had not been stained with ruthenium red. These results suggest that the particulate layer corresponds to the ruthenium-red-positive layer, which in turn corresponds to the layer visualized by interaction with antibodies against SI complex; in other words, they suggest that the SI complex is located in the particulate layer or ruthenium-red-positive layer. This interpretation is consistent with the fact that the main microvillous membrane-bound enzymes such as SI complex (3, 9, 28), aminopeptidase (10, 17), and alkaline phosphatase (16) are glycoproteins, their content of sugars being >15% (by weight) of the purified enzymes. The results shown in Figs. 1*a* and 5*a* indicate that the sugars associated with these enzymes cannot be visualized by a double staining with uranyl acetate and lead, which is able to demonstrate the glycocalyx described by Ito (7), whereas they are stainable with ruthenium red.

The appearance of the electron-opaque layer by anti-SI IgG and Fab is most probably attributed to their specific binding to their antigens on the membrane surface. Benedetti and Emmelot (1) have reported the appearance of similar but less continuous structures on the membrane surface upon interaction with antibodies. Considering the thickness (~600 Å) of the thin sections used, the continuity of the new electron-opaque layer very likely indicates that the antigens are rather close to one another on the membrane surface. From these results, however, it is impossible to decide how much of the width of the new electron-opaque layer is due to the original antigen and how much is due to bound antibodies, as have been pointed out by Parsons and Subjeck (22). In this respect, useful information can be obtained from the results of the interaction of antibodies with the purified Triton-solubilized SI complex (21). In a previous paper (21), we have reported that Triton-solubilized SI complex is an elongated molecule in which two similar subunits (45 × 65 Å) are united to each other at their long axes, that it forms an aggregate in which a certain number of elongated SI complexes align radially around a core, and that anti-SI Fab bind to various sites of aggregated SI complex molecules so that the radius of the aggregate is enlarged at most 60 Å and the fine structure of the negatively stained aggregate is greatly obscured. These effects of the antibodies on the morphology of the SI complex aggregate are very similar to their effects on the morphology of the particulate layer found in the present work. The increment in width (50 Å) of the particulate layer induced by the antibodies approximates the dimensions of Fab (35 × 65 Å) (6), quite similar to that found in the case of the Triton-solubilized SI complex aggregate (21).

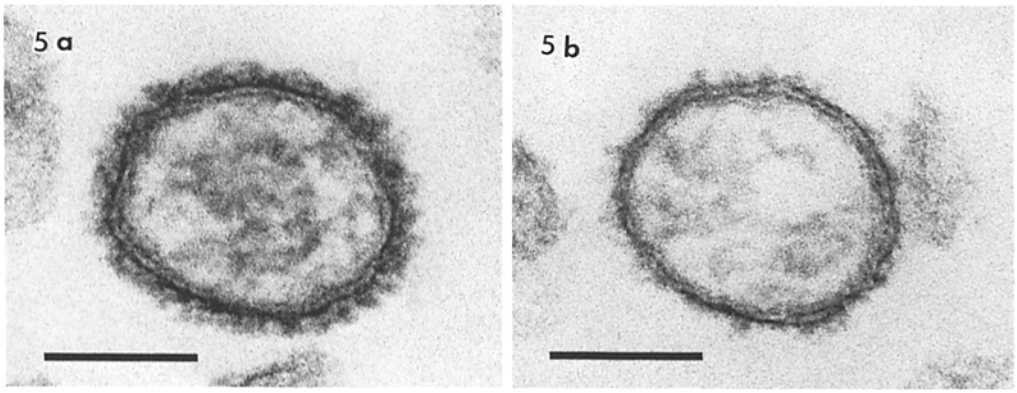

FIGURE 5 (*a*) Nontreated microvillous vesicles stained with ruthenium red. A layer ~140–150 Å wide is visualized by staining with ruthenium red. (*b*) Papainized microvillous vesicles stained with ruthenium red. Only filamentous structures can be visualized very locally on the external surface. Bars, 0.1 μm. × 200,000.

Therefore, by comparison with the results of the Triton-solubilized SI complex, it can be concluded that the SI complex molecule, including its active sites and antigenic sites, "protrudes" ~150 Å from the membrane surface. This value is an approximate one, however, because phosphotungstate most likely penetrates the membrane to some degree about which, at present, we have no data, and, furthermore, the distance of protrusion may vary to some extent depending on the quaternary structure of the SI complex, as can be expected from its different shapes in the Triton-solubilized SI complex aggregate state (see Fig. 4 of reference 21): if two molecules of the SI complex form a doughnut-shaped structure, its distance of protrusion may be smaller than that of the SI complex in a straight form. Here a few comments should be made on the possible interaction of the antibodies with sugar components of SI complex. The interaction of anti-SI IgG with purified SI complex is not inhibited by high concentration of sialic acid, hexosamines, and some neutral sugars (40). The antigenicity of the purified SI complex is inactivated by incubation at pH 5.6 or 9.4 for 30 min at 37°C (Yoshida, Akaza, Nishi and Takesue. Unpublished data). Trypsin treatment, which removes one-third of the hexosamine from the microvillous membrane, has no effect on the agglutinability of microvillous vesicles by the antibody (38). These data, together with data of Triton-solubilized SI complex (21), suggest that anti-SI IgG has little, if any, interaction with sugar components of the SI complex. Therefore, it is possible that the sugar chains associated with the SI complex extend out to more than 150 Å from the membrane surface.

We can see an interrupted gap of ~20 Å in width between the protruding particle and the membrane edge. Its signficance is unknown at present. One might think that there would be some linkage between the particle and the membrane continuum but it would be too fine to be detected in an electron microscope. Gaps of similar dimensions have been observed between each of the surrounding SI complexes and a core in the SI complex aggregate, and between the Triton-solubilized SI complex and the surface of polystyrene latex on which the complex was adsorbed (21).

Gitzelmann et al. (4, 5) found that ferritin conjugated with antibodies against the SI complex was regularly arranged at a minimum distance of 120 Å along the microvillous membrane surface, and concluded that the SI complex antigen is located at the membrane surface but does not project into the intermicrovillous space. If their conclusion is correct, anti-SI Fab molecules bound to the SI complex at the membrane surface would not exist more than 60 Å from the membrane surface, and therefore, it would be quite difficult to explain the facts that the binding of the Fab to the SI complex gives an electron-opaque layer 180 Å in width around the outer surface of the trilaminar membrane in thin sections and that it also increases the width of the particulate layer, extending from the membrane, from 150 to 200 Å in negatively stained membranes. The antibody preparation they used might have contained only antibodies against antigenic sites located nearer the membrane.

In conclusion, the SI complex is associated at its long axis with the membrane, and it protrudes ~150 Å from the outer surface of the trilaminar membrane. A good number of the particles seen on the negatively stained membrane are the SI complex.

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