

IMMUNOFLUORESCENCE AND ELECTRON MICROSCOPY OF THE CYTOPLASMIC SURFACE OF THE HUMAN ERYTHROCYTE MEMBRANE AND ITS INTERACTION WITH SENDAI VIRUS

MARTIN BÜECHI and THOMAS BÄCHI

From the Division of Experimental Microbiology, Institute for Medical Microbiology, University of Zürich, 8028 Zürich, Switzerland

ABSTRACT

A method was developed for directly observing the inner surfaces of plasma membranes by light and electron microscopy. Human erythrocytes were attached to cover slips (glass or mica) treated with aminopropylsilane and glutaraldehyde, and then disrupted by direct application of a jet of buffer, which removed the distal portion of the cells, thus exposing the cytoplasmic surface (PS) of the flattened membranes. Antispectrin antibodies and Sendai virus particles were employed as sensitive markers for, respectively, the PS and the external surface (ES) of the membrane; their localization by immunofluorescence or electron microscopy demonstrated that the major asymmetrical features of the plasma membrane were preserved. The fusion of Sendai virus particles with cells was investigated using double-labeling immunofluorescence techniques. Virus adsorbed to the ES of cells at 4°C was not accessible to fluorescein-labeled antibodies applied from the PS side. After incubation at 37°C, viral antigens could be detected at the PS. These antigens, however, remained localized and did not diffuse from the site of attachment, as is usually seen in viral antigens accessible on the ES. They may therefore represent internal viral antigens not incorporated into the plasma membrane as a result of virus-cell fusion.

KEY WORDS human erythrocytes · Sendai virus · cytoplasmic membrane surface · membrane isolation · immunohistochemistry

The characterization of structures associated with the cytoplasmic surface (PS) of plasma membranes is important for the understanding of the modulation of transmembranal cellular and viral elements expressed at the cell surface. Their involvement in virus-cell surface interactions can be postulated particularly for virus types which invade the host cell by a fusion with the plasma membrane and whose morphogenesis involves budding

at the cell surface by incorporating virus-altered plasma membrane into the viral envelope (23). A rapidly growing body of evidence points to a role for the cytoskeleton in these phenomena (6, 8, 17, 18, 20, 24). Sendai virus, used in the present study, represents a prototype for this class of viruses. They contain a viral protein (M), associated with the PS of the envelope, which plays a major role in the formation of virions by interacting with internal and external components (25).

We have developed a new technique which is suitable for the preparation of plasma membranes in which the PS is exposed and thus directly

accessible to structural analysis. Light and electron microscope observations in conjunction with conjugated antibodies to cellular and viral antigens were made on flattened membranes attached to glass or mica cover slips. This approach complements related techniques in which the attachment of cells to a substratum has been adapted for the biochemical analysis of membrane halves and of asymmetrical features of membranes (5, 9–14, 19). We report here experiments involving Sendai virus interacting with human erythrocytes as a first step in the direction of structural investigations on virus-mediated membrane alterations performed at the PS of membranes.

MATERIALS AND METHODS

Preparation of Glass and Mica

Freshly cleaned mica plates (4 × 8 mm) were used for electron microscope preparations and glass cover slips for light microscopy. The cover slips were cleaned for 2 h in 20% H₂SO₄, washed in distilled water, and finally stored in acetone for at least 2 h. The air-dried cover slips were then incubated for 24 h at 47°C with a 2% solution in acetone (dried by storage with CuSO₄) of 3-aminopropyltriethoxysilane (Aldrich Chemical Co., Inc., Milwaukee, Wis.). The cover slips were then briefly washed in 100% acetone and incubated for 1 h at 4°C with a 1% aqueous solution of glutaraldehyde. After three further washings with PBS, the cover slips were used immediately for attachment of cells.

Cells

Human erythrocytes (group O, Rh+) were prepared from blood freshly drawn into heparin solution. Cells were washed three times in PBS immediately before each experiment, and adjusted to a final concentration of 0.1% in PBS.

Membrane Preparation

The cover slips treated with the silane reagent and glutaraldehyde were covered with a few drops of a 0.1% suspension of erythrocytes which were allowed to sediment for 2 h at 4°C. The unattached cells were washed off the surface by gentle washing with PBS. The monolayer of cells was then examined by phase-contrast light microscopy. It was possible to store such monolayers for several hours at 4°C without detectable lysis or deformation of the cells. To prepare membranes of the attached cells, the cell bodies were torn off the substrate by squirting a jet of PBS at an angle of ~45° with a Pasteur pipette. The effect of the squirting was monitored by phase-contrast microscopy. The complete removal of the cell bodies resulted in the disappearance of recognizable structures on the cover slip; the membranes attached to the cover slips were not visible by this technique. The membrane preparations were stored at 4°C under PBS before being processed for immunofluorescence microscopy or electron microscopy.

Virus

Sendai virus was grown in embryonated hens' eggs and isolated from the allantoic fluid by differential centrifugation as described previously (2).

Incubation of Cells and Membranes with Virus

The cells were incubated with virus either before their attachment to the substratum or after the squirting of the attached cells. In the first case, a 1% suspension of erythrocytes was incubated with 1,000 HAU/ml of virus at 4°C for 30 min, washed with PBS at 4°C, and resuspended to the original volume. The cells were then either kept at 4°C or incubated for 15 min at 37°C (to induce virus-cell fusion) before they were allowed to sediment onto the substratum. In the second case, attached membranes were incubated with 5,000 HAU/ml of virus for 15 min at 4°C and subsequently washed three times with PBS at 4°C.

Antisera

(a) Rabbit anti-Sendai virus globulin fraction conjugated with fluorescein isothiocyanate (FITC) was the same as that used previously (4). (b) Guinea pig anti-Sendai globulin fraction conjugated with FITC was prepared from a pool of antisera obtained from animals hyperimmunized by intracutaneous injections with sucrose gradient-purified concentrated Sendai virus (grown in embryonated hens' eggs) using the same methods as for preparation of the conjugates described above. (c) Goat anti-rabbit IgG-(TRITC) (rhodamine) was a commercial preparation used without further purification (Nordic, Antwerp). (d) Rabbit antiserum was a generous gift from Dr. C. Howe (New Orleans, La.) and was the same preparation as that described previously (7). All antisera were exhaustively absorbed with human erythrocytes to remove antibodies to the cell surface before their use in the membrane experiments. (e) Guinea pig anti-rabbit IgG-ferritin conjugate was the same as that described previously (1).

Immunoreactions

Viral and/or cellular antigens present on glass cover slips were reacted with antisera or antibody conjugates for 15 min at 4°C, followed by three washes with PBS at 4°C for 5 min each. The samples were then examined immediately by immunofluorescence microscopy or processed for electron microscopy.

Electron Microscopy

Membranes fixed to mica surfaces were rinsed for a few seconds with cold distilled water, drained, and immediately frozen and stored in liquid nitrogen. The frozen preparations were then transferred onto a specimen stage precooled at -150°C of a Balzers freeze-etching apparatus (Balzers Corp., Balzers, Liechtenstein.). After evacuation to 10⁻⁶ Torr, the temperature of the specimen was elevated to -40°C and a cold trap cooled to liquid nitrogen temperature was put above the specimen for 90 min. After this sublimation period, the specimen was shadowed with platinum-carbon at an angle of 45° and with carbon at 90°. The replicas were removed from the mica surface by floating them on a 20% solution of HF and were subsequently cleaned with 20% sulphuric acid and distilled water. The specimens were examined with a Philips 201 electron microscope.

Light Microscopy

Cells and membranes attached to glass cover slips were examined using a Zeiss Invertoscope D with a Neofluar 40 phase-contrast objective. For immune fluorescence, the membrane preparations were mounted in a 50% solution of glycerol in PBS and examined with a Zeiss standard 18 microscope by incident

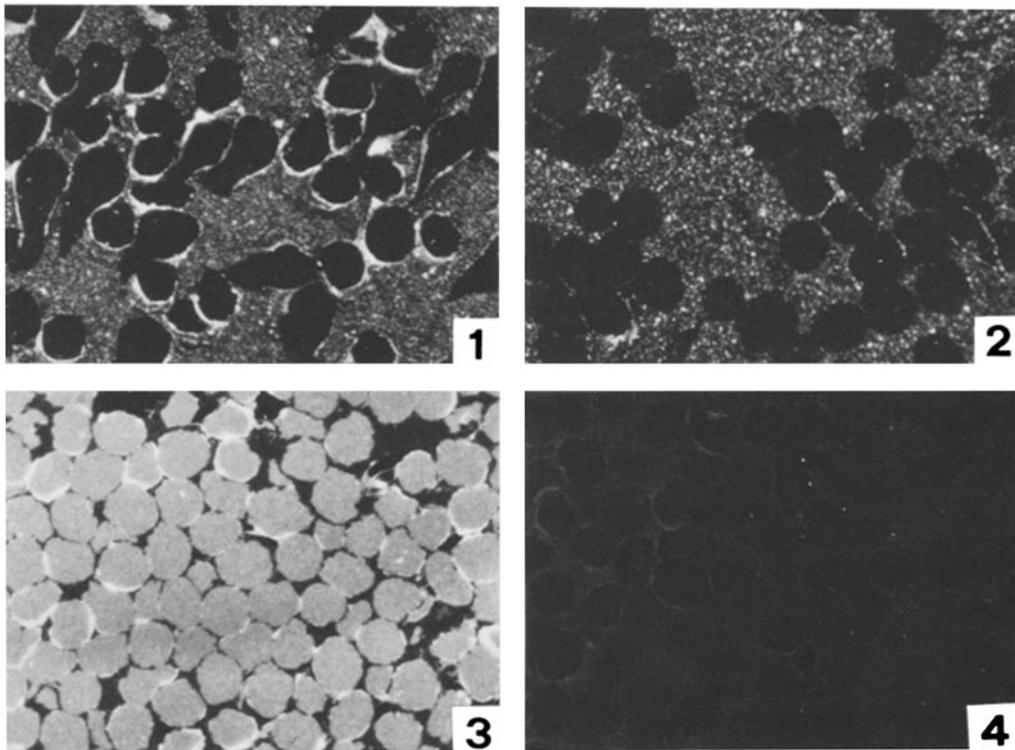
light illumination. Photomicrographs were made on Ilford FP-4 film.

RESULTS

Attachment of Cells to Cover Slips and Preparation of Membranes

The sedimentation of human erythrocytes onto the cover slips resulted in the formation of a

monolayer of cells irrespective of whether silanized mica or glass was used as a support. The arrangement of the cells was identical with that previously described (4) for cells attached to positively charged surfaces. Squirting these monolayers with a jet of PBS removed most of the cytoplasmic substance as well as the nonadherent membranes. Because the remaining flattened fragments of membranes could not be detected by



FIGURES 1-4 Glass cover slips were treated with aminopropylsilane and glutaraldehyde and subsequently coated with human erythrocytes. The attached cells were removed with a jet of PBS, leaving the membranes remaining attached to the substratum. All micrographs $\times 1,000$.

FIGURE 1 Immunofluorescence of rabbit anti-Sendai FITC reacted with membranes incubated with Sendai virus at 4°C . Bright fluorescence at peripheral zones of the membranes indicates the presence of virus particles attached to the ES exposed by a curling up of some membranes. The PS is completely unreactive with Sendai virus because of the lack of virus receptors in this area. Background fluorescence on glass helps to identify the position of the membranes.

FIGURE 2 Same as Fig. 1. In this preparation, all membranes are completely flat as compared to the preparation of Fig. 1 featuring curled-up peripheries.

FIGURE 3 Indirect immunofluorescence of rabbit anti spectrin with goat anti-rabbit IgG-TRITC. The higher intensity of fluorescence at some peripheral zones is due to membrane bilayers in curled peripheries (compare with schematic drawing, Fig. 18).

FIGURE 4 Control experiment in which membranes were incubated with goat anti-rabbit IgG-TRITC without prior reaction with specific sera.

phase-contrast light microscopy, they were visualized by reactions with fluorescein-labeled antibodies (Figs. 1–3). Alternatively, membranes were processed by freeze-drying techniques for electron microscopy and were then seen as essentially circular structures whose texture differed from that of the supporting surface (Figs. 5–7). The membranes exhibited a regular granularity on their surface, whereas the surrounding substratum was often covered with various artifactual particles which varied in size and distribution from one preparation to another (Figs. 5–11). It was noted that the adsorption of virus particles to the cells before their attachment to the support lowered the firmness of binding because, after squirting, fewer membranes were left behind (Figs. 12–17). When the glass or mica cover slips were incubated for 90 min at 20°C with ethanolamine (50 mM, pH 9.0) before the sedimentation of cells, an identical monolayer was formed. Subsequent squirting, however, in this case removed the cells together with the membranes. Similarly, efficient attachment of the cells did not occur when the support was used without pretreatment with the silane reagent and/or glutaraldehyde.

Demonstration of the Cytoplasmic Surface of the Membrane using Sendai Virus and Antispectrin as Probes

The orientation of the membranes attached to cover slip surfaces was probed with two markers characteristic for either the external surface (ES) or PS: spectrin served as a marker for PS and was visualized by specific immune reactions, whereas Sendai virus particles, by virtue of their exclusive reactivity with sialic acid residues of the ES, were visualized directly by electron microscopy or by staining with anti-Sendai FITC and served as probes for the ES. Both the images obtained by immunofluorescence techniques and electron microscopy demonstrated that most of exposed membranes belonged to the PS. Attempts to adsorb virus particles to membranes by incubating them with concentrated samples (5,000 HAU/ml) of Sendai virus (Figs. 1 and 2), when stained with anti-Sendai FITC, resulted in occasional bright rims representing ES exposed by a curling up of peripheral zones which had reacted with virus particles (see schematic drawing, Fig. 18). Analogous pictures were obtained from specimens prepared by freeze-drying and observed by electron microscopy. Virtually no virus particles were

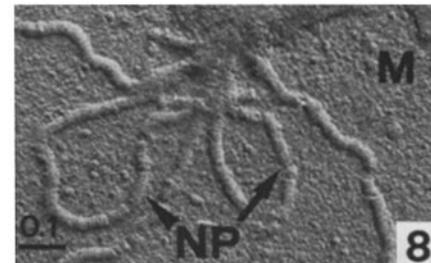
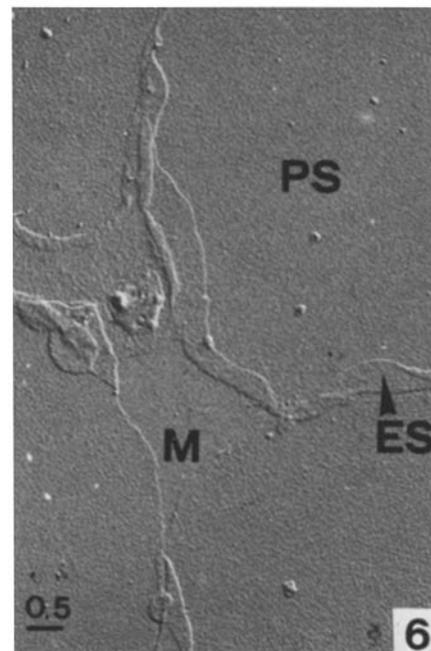
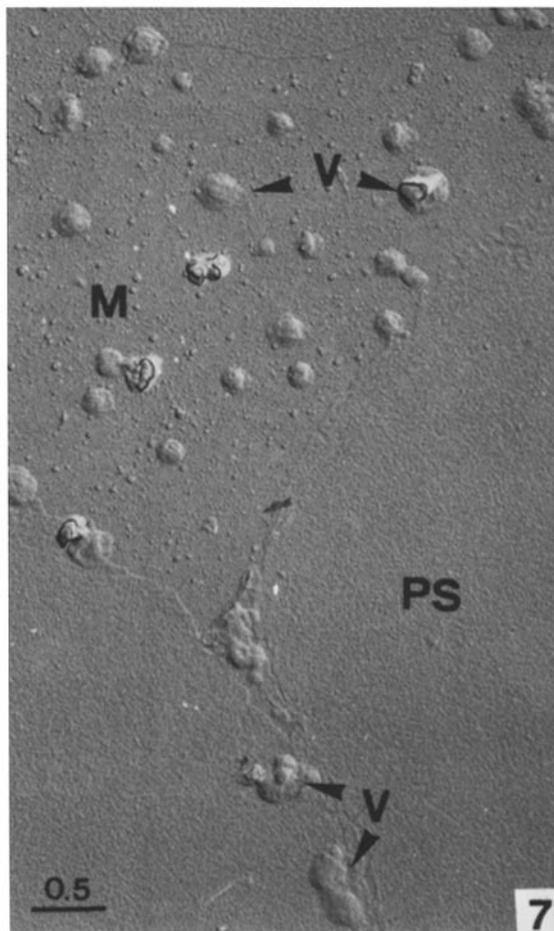
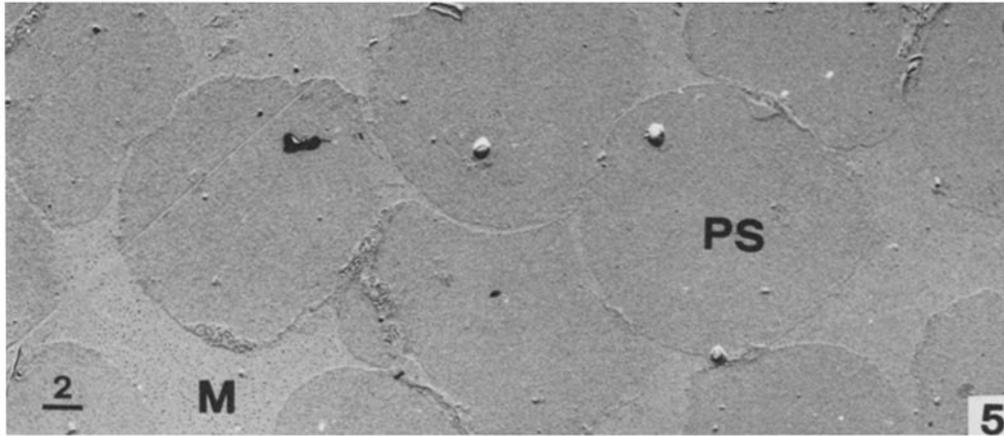
found in the central areas (PS) of the membranes. Numerous particles were, however, deposited on the substratum or were adsorbed to the ES exposed at the overlapping rims (Fig. 7).

Reactions of membranes with antispectrin and anti-IgG-TRITC yielded pictures complementary to the above situation: central areas representing the PS were fluorescent (Fig. 3), and the peripheral zones exhibited an even brighter staining with rhodamine due to the overlapping of membranes (see also Fig. 18). Control preparations reacted with anti-IgG-TRITC without prior incubations with antispectrin remained unstained (Fig. 4). Membranes reacted with antispectrin followed by guinea pig anti-rabbit IgG-ferritin and processed for electron microscopy displayed ferritin granules at their PS, whereas the ES remained smooth (Figs. 10 and 11). The ferritin conjugate was not reactive with membranes preincubated with PBS instead of antispectrin (Fig. 9).

Sendai Virus-Membrane Interaction

The prototype interaction of Sendai virus with the cell surface (i.e. adsorption and fusion between the viral envelope and the plasma membrane) was studied “from within” by preparing membranes of cells preincubated with virus. Depending on whether the virus was allowed to interact with cells for 30 min at 0°C exclusively or at 0°C followed by an incubation for 15 min at 37°C, different patterns of immune reactions were demonstrable on the ES of attached, unfixed membranes. Virus adsorbed to cells at 0°C was detectable by immunofluorescence at peripheral zones of the membranes (ES), whereas the central areas representing PS were fluorescent only in some cases (Fig. 12). Cells incubated with virus at 37°C, however, always exhibited fluorescent spots in PS areas (Fig. 13). This latter staining pattern was identical with that seen on membranes reacted with anti-Sendai FITC conjugates after a fixation with acetone (Figs. 14 and 15).

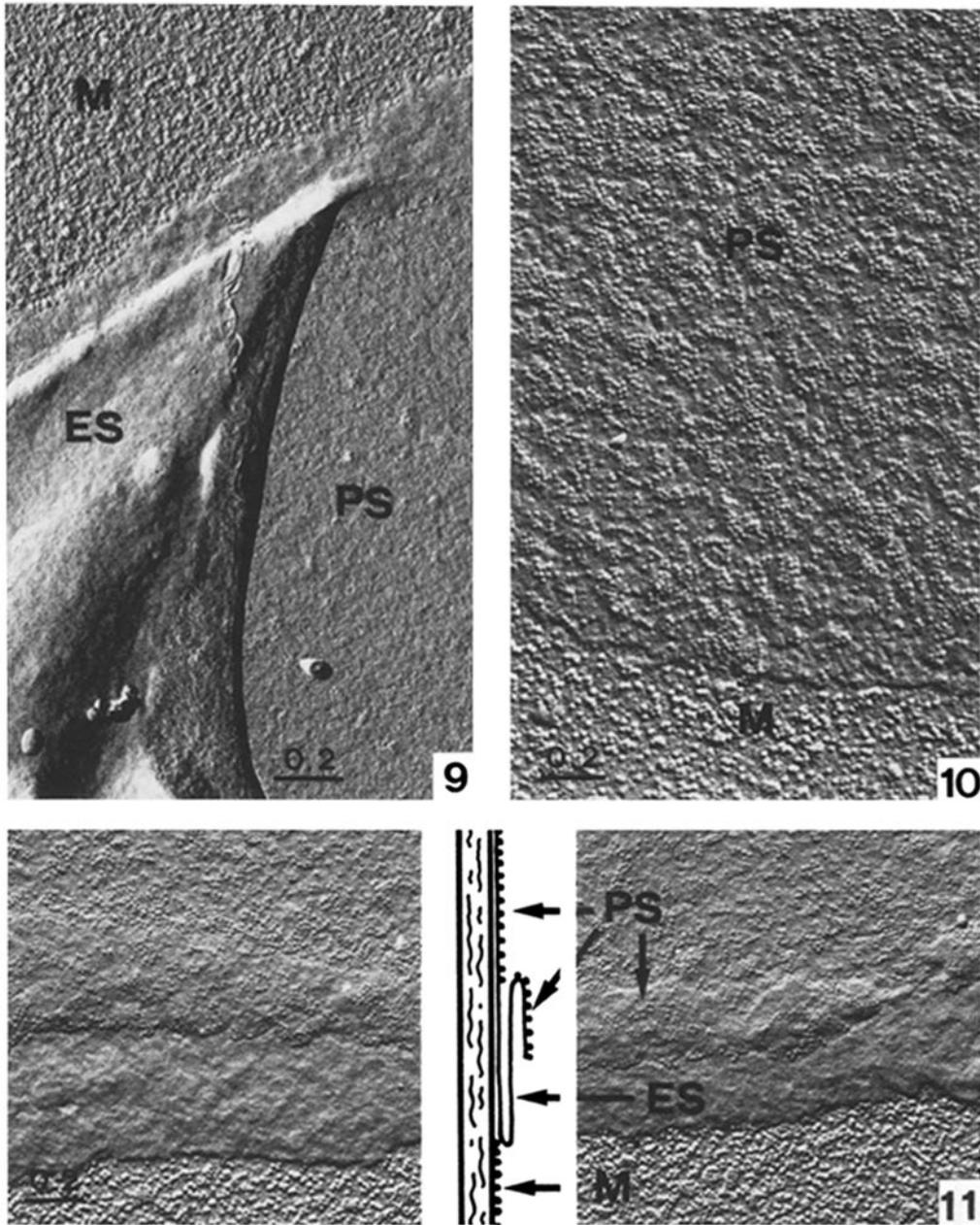
Double-staining procedures were employed for the simultaneous observation of viral and cellular antigen on the same preparation. Membranes prepared from cells with virus adsorbed at 4°C were stained with a sequence of reactions involving guinea pig anti-Sendai-FITC, rabbit antispectrin-TRITC and guinea pig anti-rabbit IgG-FITC. The staining with anti-viral antibodies (Fig. 16) was identical with that obtained after single-labeling procedures (Fig. 12). Occasionally, areas showing



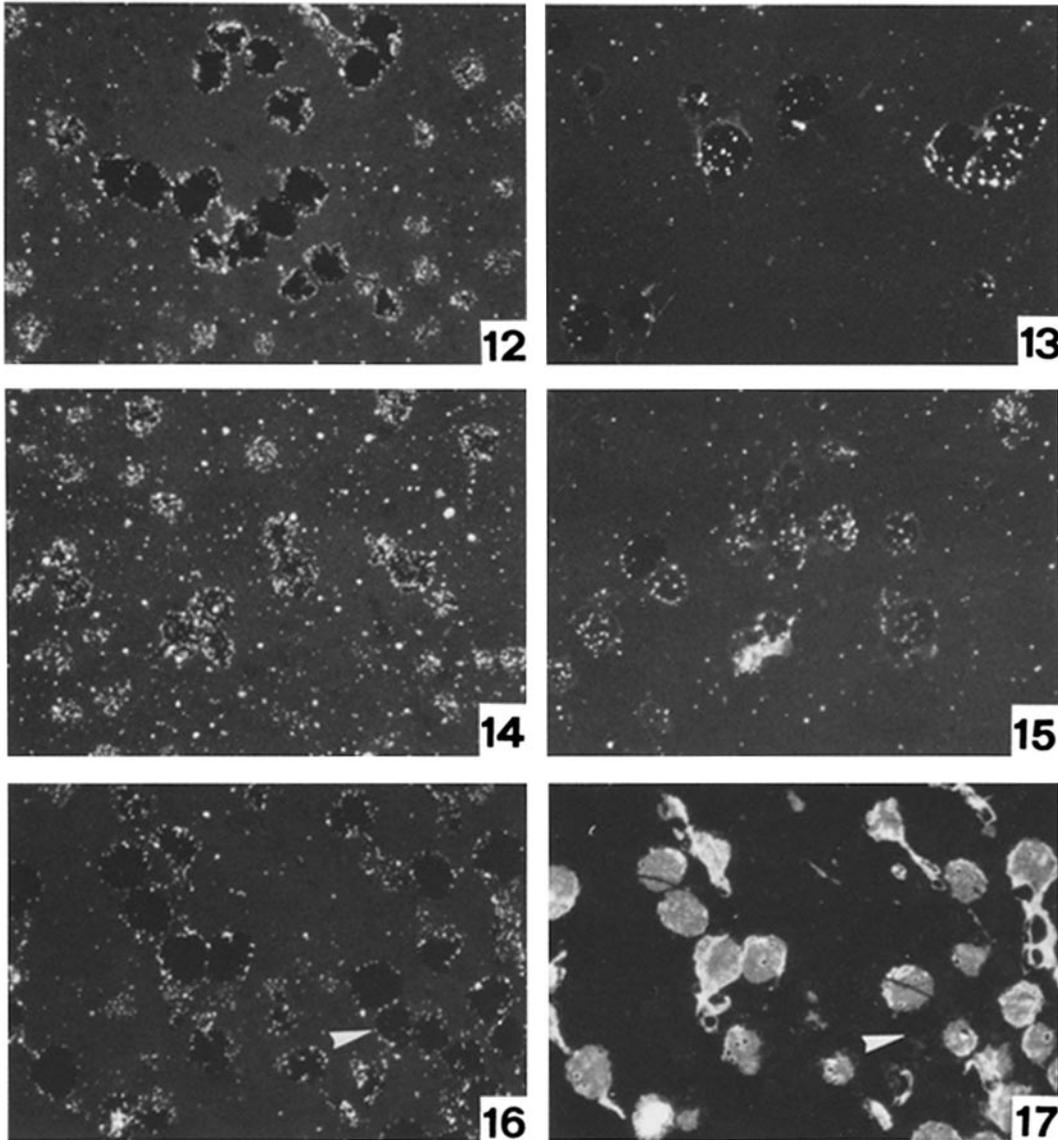
FIGURES 5 and 6 Examples of membrane preparations on mica surfaces (*M*) processed for electron microscopy by freeze-drying and shadowing with Pt/C. The membranes are oriented to expose their cytoplasmic surface (*PS*) except in some peripheral areas representing curled-up membranes exposing the external surface (*ES*). Bar indicates scale in micrometers.

FIGURE 7 Attached membranes were incubated with concentrated preparations of Sendai virus particles (*V*) which adsorbed exclusively to the peripheral zones representing *ES* (compare Fig. 6) bearing the virus receptors of the membrane. The cytoplasmic surface (*PS*) of the membrane was completely unreactive. Numerous virus particles have sedimented onto the substratum (*M*) and are discernible as spherical structures with a diameter of 200–400 nm.

FIGURE 8 Same as Fig. 7. The higher magnification documents the high resolution of the freeze-dried preparations by the visualization of nucleoprotein subunits (*NP*) released from a disrupted virus particle.



FIGURES 9-11 The attached membranes were allowed to react with a sequence of immune reactions with rabbit antispectrin and guinea pig anti-rabbit IgG ferritin (Figs. 10 and 11) or with anti-rabbit IgG ferritin alone in control experiments (Fig. 9). Antispectrin was localized by the ferritin conjugate exclusively on the PS, as indicated by the presence of ferritin granules on the internal face and the smooth appearance of the ES. One PS area seen on Fig. 11 represents the uppermost surface exposed by a folding of the membrane in form of a Z (see schematic *inset*). The granularity of the background which is also seen in controls (Fig. 9) is probably due to non-specific adsorption of the conjugates to the substratum.



FIGURES 12-17 Immunofluorescence of membranes prepared from cells preincubated with Sendai virus for 30 min at 4°C (Figs. 12, 14, 16, 17) or at 4°C followed by 10 min at 37°C (Figs. 13 and 15). All micrographs $\times 1,000$.

FIGURE 12 Viral antigen is recognizable as granular spots at the periphery of the attached membrane. The central areas (PS) are usually free of fluorescence, indicating that the virus particles are localized at the ES and not accessible to the anti-Sendai FITC.

FIGURE 13 Membranes of cells with virus fused with the plasma membrane by incubation at 37°C. The penetration of the cell surface by viral antigens is recognizable by the presence of granular spots on the PS.

FIGURE 14 Same preparation as in Fig. 12. Before the immunoreaction, however, the sample was fixed with acetone. The virus particles adsorbed to the ES are tagged with antibodies, producing a granular fluorescence.

FIGURE 15 Same as Fig. 13, after acetone fixation.

FIGURES 16 and 17 Membranes of cells treated with Sendai virus at 4°C before their adsorption to the glass surface and the preparation of membrane. The sample was double stained with a sequence of immunoreactions using guinea pig anti-Sendai-FITC, rabbit antispectrin and goat anti-rabbit IgG-rhodamine. The distribution of the viral antigen is identical with that of Fig. 12. The staining of spectrin, however, reveals that not all viral antigens are associated with membranes (see arrow on Fig. 17, for instance) because they were removed during the course of the preparation.

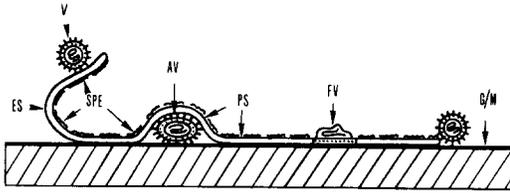


FIGURE 18 Schematic representation of the orientation of membranes of cells attached to silanized cover slips and squirted with PBS. The membranes are attached with their external surface (ES) to the substrate and are accessible to probes at sites which are exposed by a curling of peripheral zones of the membrane. The cytoplasmic surface (PS) is oriented to the surface of the preparation and is covered by the peripheral spectrin (SPE). Depending on experimental conditions, Sendai virus particles are associated with the membrane in three different fashions: (a) adsorbed to the ES by incubation of cells with virus at 4°C before their adsorption and the preparation of membranes (AV); (b) adsorbed to the ES by incubation of attached membranes (V); and (c) virus adsorbed and fused with cells at 37°C before their attachment and the preparation of the membranes (FV).

viral antigens (Fig. 16, arrow) appeared devoid of spectrin (compare with identical area on Fig. 17). This suggests that some membranes were detached during the staining procedure while viral antigen remained on the glass.

DISCUSSION

The direct observation of structures on the PS of plasma membranes represents a further application of the strategy to characterize surfaces of cells after their attachment to a support. A variety of techniques suitable for the binding of cells or membranes to positively charged surfaces have already been proposed (5, 9, 10, 12–14, 16, 19). An alternative but more tedious method based on the covalent coupling of polylysine to succinylamino-propyl glass has also been employed (15).

For our experiments, a modification of a principle proposed by Robinson et al. (22) to couple cells covalently to aminoalkylsilane glass or mica was used. This not only allowed the preparation of flat membranes with their PS exposed after the body of the cells had been torn off by squirting with a jet of physiological fluid, but it also proved suitable for high-resolution morphological analysis by light and electron microscopy. The exclusive occurrence of sialic acid residues bearing receptor activity for Sendai virus at the ES and of spectrin as peripheral protein at the PS offered sensitive

markers for the visual identification of the orientation of the membrane fragments.

Previous studies employing noncovalent binding procedures had shown that the attachment of cells to glass beads does not disrupt the organization of the membranes (5, 10, 13, 14). The specific binding of antispectrin antibodies and Sendai virus particles, respectively, to exposed surfaces (see schematic drawing of Fig. 18) showed that the asymmetry of the membrane was preserved by our preparation method. While our technique allows the specific immunological and structural characterization of elements associated with the PS, the small amounts of material involved were not suitable for biochemical analysis. Accordingly, the reactive groups on the support and on the plasma membranes responsible for the binding of the cells could not be further characterized. The prevention of firm binding of the cells upon preincubation of the cover slips with ethanolamine points to an important role of glutaraldehyde as a mediator in the reaction.

The immune reactions were highly specific at the levels of both light and electron microscopy. Anti-IgG antibodies conjugated with rhodamine or ferritin reacted exclusively with the PS of membranes preincubated with antispectrin sera (Figs. 3, 10, and 11). Sendai virus tagged with anti-Sendai-FITC and recognizable by a granular fluorescence was associated with both the ES and the PS of the membranes, representing, respectively, adsorbed virus particles and virus particles which had penetrated the membrane by fusion. In experiments in which virus was added to the membrane after squirting (Figs. 1 and 2), the fluorescence detectable on the cover slip surface represented virus particles which were deposited there by sedimentation. Virus particles which became detached from the cells during the preparation of membranes were also found on the support (Figs. 12–16). No effort was made, however, to find conditions blocking the nonspecific interaction of viral material with the cover slip surface because this background did not influence the specificity of the reactions of antibodies and/or virus particles within membrane areas. Moreover, the background helped to define the edge of the membranes, the location of which would have been difficult to demonstrate otherwise.

A different type of background consisting of granules of variable size was occasionally encountered in freeze-dried preparations examined by electron microscopy; with the use of dried acetone

for the dilution of the silane reagent, these structures, probably representing polymerized silane and/or ice crystals, were largely eliminated (Figs. 5–11).

Preliminary experiments with nucleated cells of complex surface morphology including microvilli have shown that the binding forces between the substratum and cell surface are firm enough to render the same technique useful. This does not contradict our present observation that the virus particles adsorbed to the erythrocyte surface lowered the ability of cells to attach during the preparation (Figs. 12–17). In fact, erythrocytes, by virtue of their rigidity and biconcave shape, may be less prone to adjust their surface in response to deformations introduced by adsorbed virus particles.

In contrast to the examination of the membranes by immunofluorescence which involved incubations with sera and buffers adjusted to physiological conditions, electron microscope procedures required a brief wash in distilled water, followed by freezing. Freeze-drying procedures followed by Pt/C shadowing yielded replicas with a high resolution of details which allowed the easy identification of ferritin-labeled antibodies (Figs. 10 and 11) and of certain viral elements such as the nucleoprotein (Fig. 8).

The study of penetration of Sendai virus through the membrane as observed “from within” represents the first application of this technique. The interaction of Sendai virus with human erythrocytes includes several discrete steps (3), two of which were observed in our experiments: (a) the adsorption of viral particles to the ES at 4°C, and (b) the penetration of the membrane by fusion of the viral envelope with the plasma membrane. Ambiguities with respect to the orientation of the membrane and the relative position of viral and cellular antigens were ruled out by immunofluorescence double-labeling techniques allowing the simultaneous localization of two different sets of antigens on the same preparation (Figs. 16 and 17). With immunofluorescence labeling, we have shown that virus adsorbed at 4°C to the ES before the attachment of cells did not penetrate the membrane (see Figs. 12, 16, and schematic representation, Fig. 18). This predictable result is a strong indication that the preparation of the membrane did not disrupt its integrity. After acetone fixation, however, the permeability of the membrane was altered, allowing antibodies to penetrate to the viral antigens trapped between membrane and

glass (Fig. 14). Fusion from without of the viral envelope with the plasma membrane was monitored at the PS by the appearance of fluorescence spots (Fig. 14). This finding contrasts with the rapid diffusion of viral antigens upon virus-cell fusion shown in previous studies (2–4). The viral elements expressed at the ES, however, must represent sets of antigens different from those exposed after virus-cell fusion at the PS. The antiserum contained activities against several major antigens of Sendai virus, i.e. antibodies to the external envelope glycoproteins (HN,F) as well as to the core proteins (NP,M) (21). The internal antigens (M and NP) would be expected to show a patchy distribution because they obviously would not become integrated into the plasma membrane structure as a result of virus-cell fusion and, consequently, should remain stationary at the primary site of adsorption of the virus particle to the cell rather than diffusing in the plane of the membrane. Further definition and differentiation of structures expressed at the PS and ES, however, will depend on the availability of monospecific antisera to the various viral proteins.

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