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REPLICA-IMMUNOGOLD TECHNIQUE APPLIED TO STUDIES ON MEASLES VIRUS MORPHOGENESIS

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Abstract

The replica technique was applied to studies on the dynamic process of measles virus budding on infected HeLa cells. Virus structures were identified by labeling with anti-measles antibodies and protein A-gold. The combination of these two methods enabled us (1) to characterize the sequence of virus budding at the plasma membrane, (2) to localize virus structures on cytoskeletons of infected cells, and (3) to study the influence of Ca^{2+} ions on virus structures at the plasma membrane. Studies on platinum carbon surface replicas suggest that the process of virus budding is similar to the genesis of cellular microvilli. Replicas prepared from cytoskeletons of infected cells reveal a close association of budding virus with actin filaments composing the outer parts of the networks. Replicas of apical plasma membranes isolated from infected cells show the attachment of viral nucleocapsids to the protoplasmic membrane face of infected cells. These nucleocapsids are not present on membranes prepared from cells treated with calcium and the ionophore A23187. In addition viral cell surface antigens become randomly distributed on these cells. The data suggest that measles virus morphogenesis at the plasma membrane of cultured cells is dependent on the function of the cytoskeleton and may be influenced by Ca^{2+} ions.

KEY WORDS: Replica-immunogold technique, virus budding, cell surface, cytoskeleton, apical plasma membranes.

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Introduction

The infection cycle of paramyxoviruses terminates with the assembly of structural virus components at the plasma membrane (Simons and Garoff, 1980; Dubois-Dalcq et al., 1984). The assembled virus particles are then released from the cell by budding out from the plasma membrane. In the course of this step the viral particles gain their lipid envelope, which contains virus-specific transmembrane glycoproteins. The forces responsible for the formation of viral buds at the plasma membrane are not known. They may either result from a self-assembly process of viral proteins (Hewitt, 1977; Hewitt and Nermut, 1977), or may be induced by their interaction with cellular structures, most probably the cytoskeleton. The latter may be assumed due to the fact that released viral particles contain actin (Wang et al., 1976) and that viral antigens at the cell surface co-migrate with actin into patches upon treatment of infected cells with cytochalasin B (Sundqvist and Ehrnst, 1976).

In recent years we focused on budding of measles virus, a member of the paramyxovirus family. Budding of this virus begins with the attachment of viral nucleocapsids to the protoplasmic face of the plasma membrane. Binding of the nucleocapsids is dependent on the presence of the viral M-protein (Yoshida et al., 1976) and takes place at membrane areas, where viral glycoproteins are inserted (Dubois-Dalcq and Reese, 1975).

We intended to clarify this dynamic process by investigating the interaction of the viral components with cellular structures, specifically the plasma membrane and the underlying cytoskeletal network. As budding of viruses is a morphologically detectable phenomenon, the application of light and electron microscopic methods seemed to us to be a most suitable way to understand its mechanism. For electron microscopic studies we used the replica method and combined it with immunogold labeling (Horisberger, 1981; Roth,

1983). The combination of replica technique with immunocytochemical labeling has successfully been employed for the detection of cellular receptors (Tillack et al., 1971; Robenek et al., 1983; Robenek and Severs, 1984) and viral antigens (Mannweiler et al., 1982; Odenwald et al., 1986) at the plasma membrane as well as for the identification of cytoskeletal components (Lawson, 1984; Hartwig and Shevlin, 1986). The use of colloidal gold and ferritin have also been described for both pre- and post-fracture labeling (Pinto da Silva and Branton, 1970; Pinto da Silva et al., 1981) as well as for post-shadow labeling (Rash et al., 1982). Special equipment, such as the replica detachment and the automatic washing device (Hohenberg and Mannweiler, 1980; Hohenberg et al., 1986) developed in our laboratory enabled us to prepare large and stable stereo replicas from cultured cells grown on glass coverslips. To each of the questions arising in the course of our studies the replica technique was specifically adapted, which allowed us (1) to follow the sequence of virus budding at the cell surface, (2) to identify the interaction of viral structures with the cytoskeleton, and (3) to study the influence of Ca^{2+} ions on budding virus structures.

Materials and Methods

Cells and viruses

HeLa cells grown on glass coverslips were used throughout the studies. The cells were cultivated with minimum essential medium (MEM) supplemented with 5% fetal calf serum. Cells were infected with the Edmondston strain of measles virus at an input multiplicity of 0.1 plaque forming units (PFU) per cell.

Antibodies

Labeling of viral structures was done with a polyclonal rabbit anti-measles antiserum, or with a monoclonal antibody to measles virus hemagglutinin. The specificity of these antibodies was shown previously (Bohn et al., 1982).

Protein A-gold

Colloidal gold of 12 nm in size was prepared according to Frens (1973) by adding 5 ml of 1% sodium citrate to 100 ml of boiling 0.01% tetrachloroauric acid. Colloidal gold was coated with protein A (Romano and Romano, 1977) as described by Horisberger and Clerc (1985), stabilized with polyethylene glycol (PEG) and made isotonic by addition of K-phosphate buffer (pH 7.4).

Preparation of intact cells

Infected cells were washed briefly with 0.2 M phosphate buffer (pH 7.4), fixed with a mixture of 1% paraformaldehyde and 0.025% glutaraldehyde in this buffer for 20 min and rinsed with buffer for another 20 min. They were then labeled with antibodies and with protein A-gold at room temperature for 20 min each and were postfixed with 1% OsO_4 for 1 h. The probes were dehydrated through a graded series of ethanol, passed through Freon 113 and critical point dried with Freon 13.

Preparation of cytoskeletons

The cytoskeletons were prepared as described recently (Bohn et al., 1986). The cells were briefly rinsed with phosphate buffered saline (PBS)(pH 7.2) and extracted with an ice-cold buffer composed of 20 mM imidazole, 80 mM KCl, 2 mM $MgCl_2$, 2 mM EGTA, 1% NP40 and Trasylol (100 U/ml) for 5 min. The cytoskeletons were rinsed with the same buffer containing no detergent for another 5 min. In some preparations the cytoskeletons were then incubated with heavy meromyosin(HMM)(Sigma, Munich, F.R.G.) at a concentration of 2 mg/ml for 20 min at room temperature. Cytoskeletons were fixed with 1% paraformaldehyde and 0.025% glutaraldehyde in imidazole buffer for 20 min, rinsed with buffer for 20 min, and were labeled with antibodies to hemagglutinin and with protein A-gold for 20 min each. They were dehydrated with ethanol and critical point dried.

Preparation of apical plasma membranes

Optimal conditions for the isolation of apical plasma membranes of cultured cells were described previously (Rutter et al., 1985). The cells were rinsed with PBS and then incubated with rabbit anti-measles antibodies and with protein A-gold for 10 min each at 0^oC. The cultures were then incubated with an 'internal buffer' composed of 100 mM KCl, 5 mM $MgCl_2$, 3 mM EGTA, 20 mM K phosphate buffer (pH 6.8), 0.1% tannic acid for 2 min at 0^oC. After a short washing with this buffer containing no tannic acid, the cultures were covered with a second glass coverslip coated with alcian-blue and were gently pressed together in the cold for 30 min. The coverslips were separated from each other in 'internal buffer'. The upper coverslip with adhering plasma membranes was immersed in 'internal buffer' containing 1% glutaraldehyde for 1 h. They were postfixed with 1% OsO_4, dehydrated in ethanol and critical point dried.

Preparation of replicas

Shadowing of the critical point dried probes, as well as floating, cleaning and mounting of the replicas were discussed and described in detail in a previous paper by Hohenberg et al. (1986). Intact cells as well as apical plasma membranes were shadowed with 2.0-2.5 nm platinum/carbon at a fixed angle of 45^o and with 15.0-20.0 nm carbon at a 90^o angle. Cytoskeletons were rotated at an angle smaller than 30^o while shadowed with platinum/carbon or with carbon alone and

thereafter stabilized by shadowing with carbon at a 90° angle. Shadowing was done in a Bioetch 2005(Leybold-Heraeus, F.R.G.) at -100°C and a pressure of 5 x 10^{-8} mbar. The shadowed probes were detached from the glass by floating on 10% hydrofluoric acid and were then transferred onto distilled water for 5 min. Only surface replicas which were prepared from intact cells were cleaned from biological material with 30% chromic acid. All replicas were mounted on grids without supporting films and examined in a Philips EM 400 T.

Results

Dissection of the budding sequence at the plasma membrane

Virus specific structures first become visible at the cell surface at about 18 h post infection. By use of the replica technique three distinct viral structures could be observed on infected HeLa cells (Fig. 1a), these are spherical virus particles, strand-like structures, and stub-like protrusions. These structures could be identified on replicas by immunogold labeling with antibodies to measles virus hemagglutinin and protein A-gold (Fig. 1b). The labeling also showed that this virus-specific cell surface protein was always associated with the above mentioned structures and was never distributed in a diffuse manner at the cell surface.

In order to clarify the formation of these structures, we looked for drugs which could influence the process of budding. Phenothiazines, known as Ca^{2+}-calmodulin inhibiting drugs (Weiss et al., 1980), were shown to be able to influence processes at the plasma membrane, such as endo- and exocytosis (Elferink, 1979; Salisbury et al., 1980), as well as the cell surface morphology (Osborn and Weber, 1980).

Addition of one of the two phenothiazines, trifluoperazine or chlorpromazine to the culture medium of infected cells at 18 h post infection prevented the formation of spherical virions and led to a disappearance of cellular microvilli (Fig. 2a). The minimal drug doses inducing this effect were 10 µM trifluoperazine and 30 µM chlorpromazine respectively (Bohn et al., 1983). Immunogold-labeling with antibodies to hemagglutinin indicated that virus-specific strands had accumulated at the cell surface (Fig. 2b). These strands showed the same patchy distribution of hemagglutinin as was found on non-treated infected cells. Transfer of drug-treated cells into drug-free medium restored the normal cell surface morphology within 1 h at 37°C (Bohn et al., 1983). Cellular microvilli appeared again and the virus-specific strand-like structures formed stub-like processes at the cell surface.

To study if the phenothiazines affected the interaction of viral structures with the cytoskeleton, we incubated phenothiazine-treated infected cells with cytochalasin B. As shown by immunofluorescence (Bohn et al., 1983) cytochalasin B induced redistribution of actin into patches, but comigration of viral surface antigens with these actin patches, normally visible on cytochalasin B-treated cells (Sundqvist and Ehrnst, 1976), was prevented by phenothiazines.

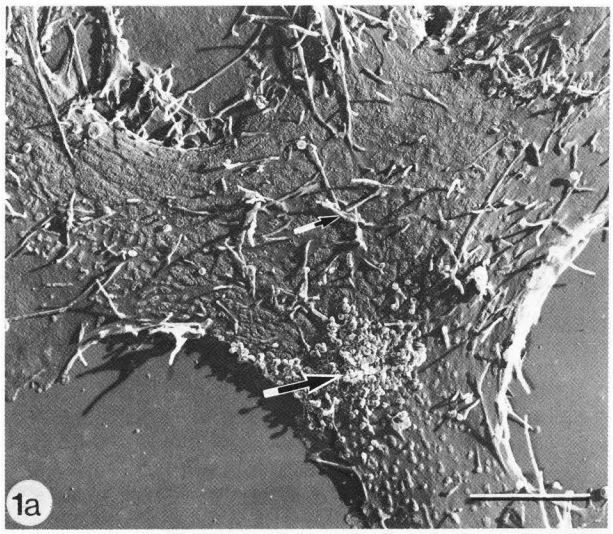
The data indicated that budding of measles virus could be dissected by use of phenothiazines. Budding could be shown to start with the formation of strand-like structures, which leave the plane of the plasma membrane at one end, forming stub-like protrusions. These protrusions vesiculate, leading to the appearance of spherical virions at the cell surface. The results also led us to assume that the cytoskeleton is involved in the formation of viral buds at the plasma membrane.

Association of viral structures with the cytoskeleton

In order to clarify the role of cytoskeletal structures in the process of budding we prepared cytoskeletons of infected cells by use of the non-ionic detergent NP40. These cytoskeletons proved to retain the majority of viral polypeptides, including the envelope protein hemagglutinin. Infective units were present even in the cytoskeletal fraction (Bohn et al., 1986).

Cytoskeletons shadowed with platinum and carbon exhibited a complex three-dimensional organization of filaments in TEM (Fig. 3a). Numerous electron dense structures were located on the outer parts of the network (Fig. 3b). Viral structures were identified by labeling with antibodies to hemagglutinin and protein A-gold. Identification of the gold-label was difficult on cytoskeletons shadowed with platinum (Fig. 3b), and could far better be visualized on cytoskeletons only shadowed with carbon (Fig. 4). Stereo images also proved that the labeled virus structures were not trapped within the network, but were associated with filaments composing the outer part of the network (Fig. 4). All stages of virus budding normally visible at the cell surface could be detected, including strand-like structures, protrusions emanating from these strands, and spherical virus structures (Bohn et al., 1986).

Cytoskeletons were incubated with heavy meromyosin to identify actin filaments in these networks. Platinum and carbon shadowed cytoskeletons now revealed that filaments which were in contact with viral structures were irregularly shaped and thickened to 230 Å, suggesting that they represented actin



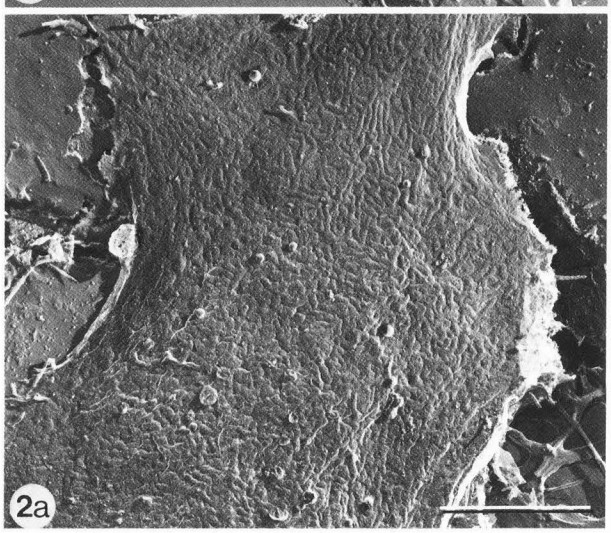


Fig. 1. Platinum/carbon surface replicas of measles virus infected HeLa cells (28 h post infection). (a) At low magnification the cells reveal cellular microvilli (➔) and accumulations of budding virus structures (➔). (b) Identification of viral structures by labeling with antibodies to hemagglutinin and protein A-gold; strand-like virus structures (➔), stub-like protrusions and spherical virions (➔), microvilli (⇨). Bar (a) = 5 µm; (b) = 1 µm.

Fig. 2. Platinum/carbon surface replicas of infected cells treated with trifluoperazine (20 µM) from 18 to 28 h post infection. (a) Microvilli and budding virions are absent; strand-like virus structures have accumulated at the cell surface. (b) Labeling of strand-like virus structures as in Fig. 1b. Bar (a) = 5 µm; (b) = 0.5 µm.

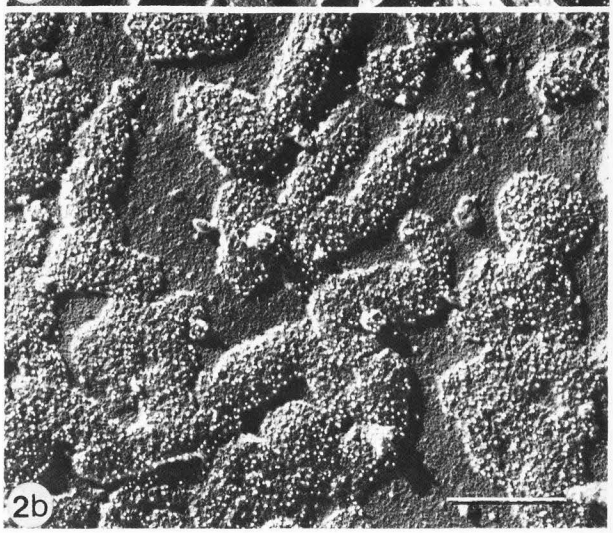
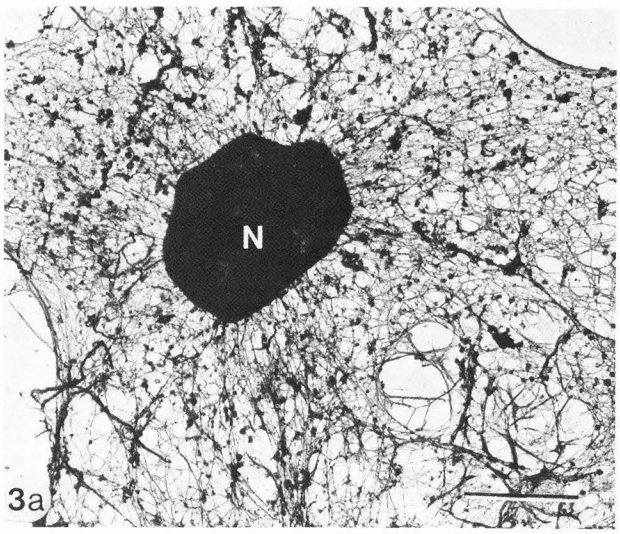
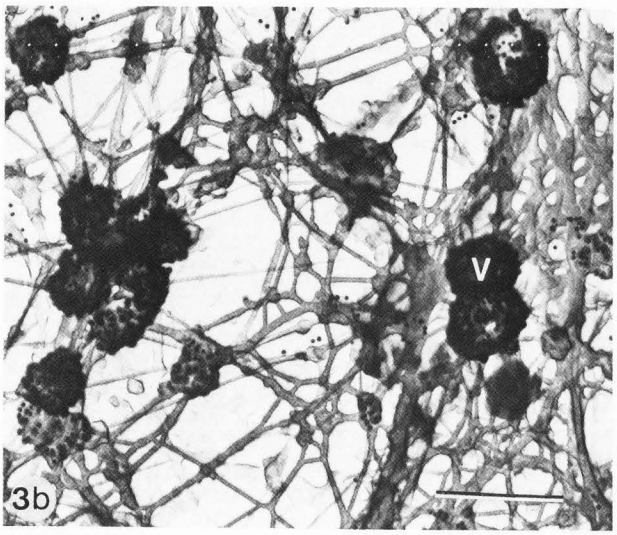
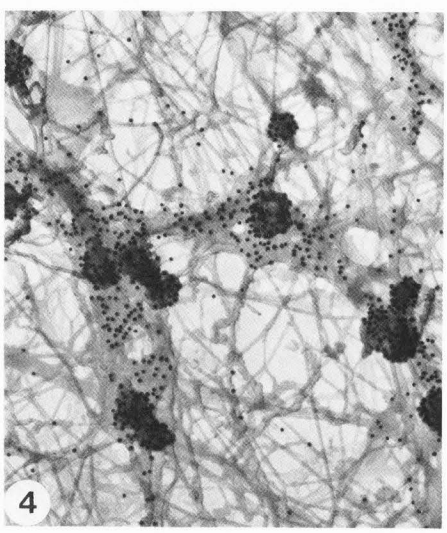


Fig. 3. Cytoskeletons of infected cells rotary shadowed with platinum/carbon. (a) Numerous electron dense particles are scattered on the cytoskeletons. (b) Identification of viral structures (V) by immunogold-labeling as in Fig. 1b; Nucleus (N). Bar (a) = 5 µm; (b) = 0.5 µm.

filaments (Fig. 5). Smooth undecorated filaments were found within deeper parts of the cytoskeletons (Fig. 5). Ultrathin sections of flat embedded extracted cells, which had been incubated with heavy meromyosin, proved that actin filaments emanated into budding virus structures with the barbed ends and were in close association with viral nucleocapsids (Fig. 6).







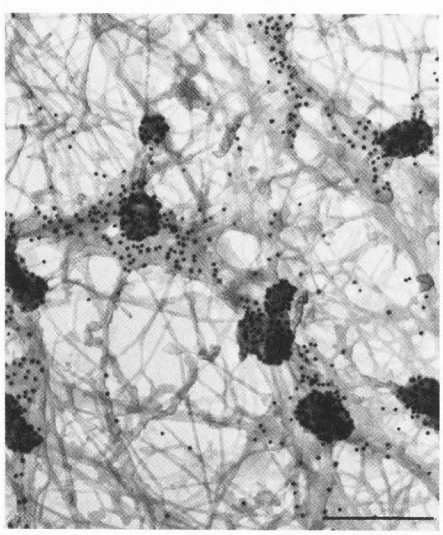
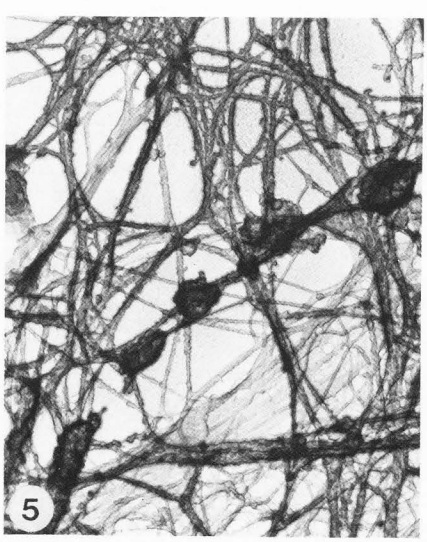


Fig. 4. Stereo images of a cytoskeleton rotary shadowed with carbon; labeling of virus structures as in Fig. 1b. Virus structures emanate from the outer part of the filament network. Bar = 0.5 µm.



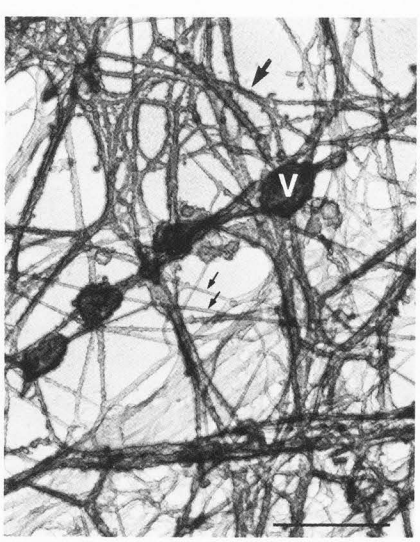


Fig. 5. Stereo images of a cytoskeleton incubated with heavy meromyosin and rotary shadowed with platinum and carbon. Filaments composing the upper part of the network in the vicinity of virus structures (V) are irregularly shaped (➡). Smooth filaments extend through deeper parts of the network (⟶). Bar = 0.5 µm.

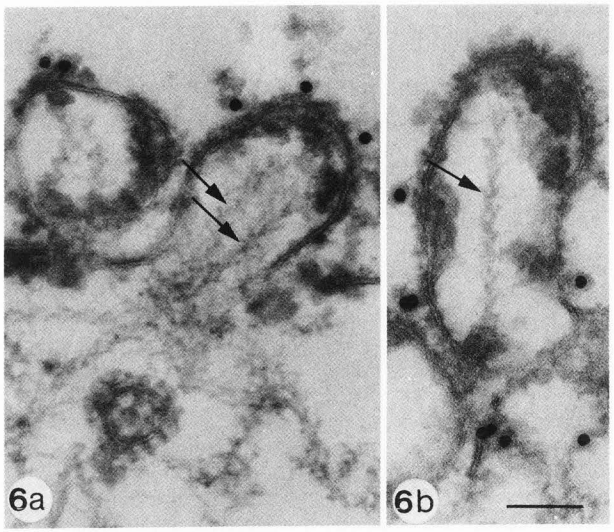
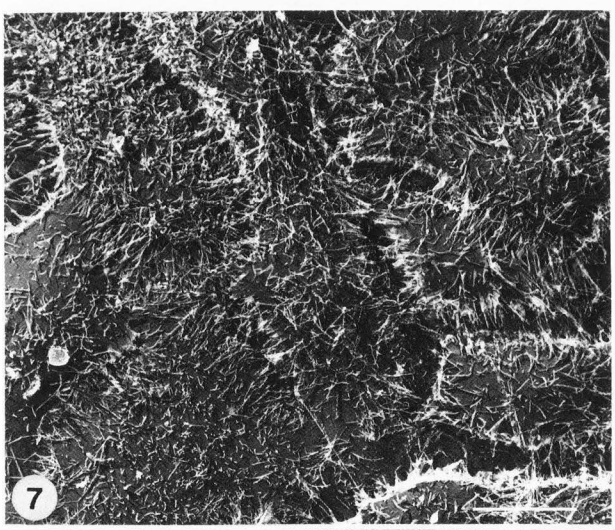
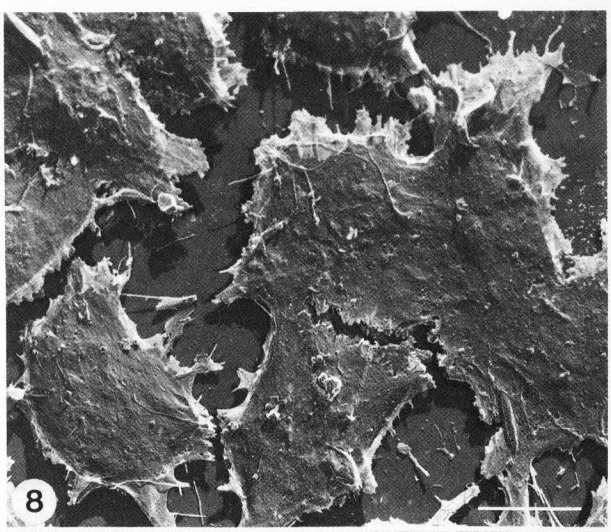


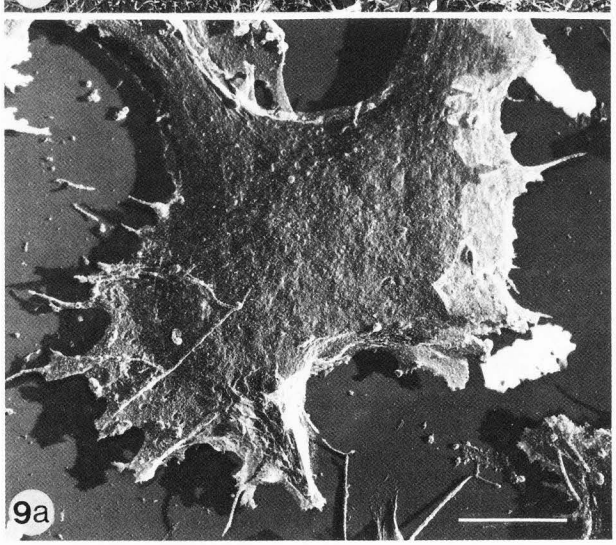
Fig. 6. Ultrathin sections of budding virions. Actin filaments, which were decorated with heavy meromyosin emanate into budding virions; labeling of virus particles with a rabbit anti-measles antiserum and protein A-gold; decorated actin filaments (⟶). Bar (a) and (b) = 0.1 µm.

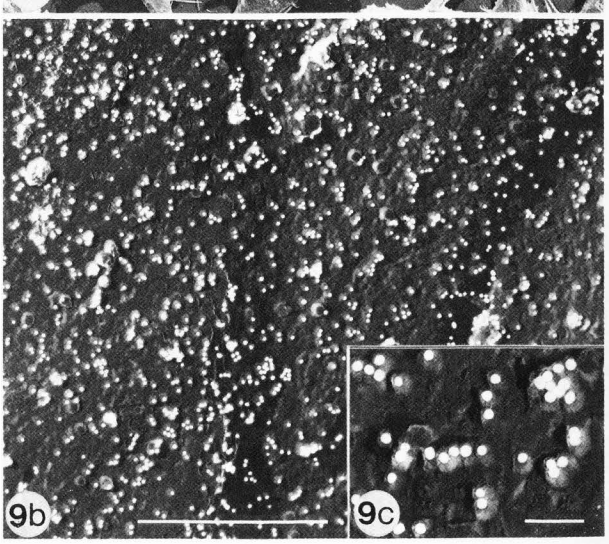
Fig. 7. Platinum/carbon surface replica of infected cells treated with the calcium ionophore A23187 (10 µm) in the presence of 1 mM $MgCl_2$. Cells show the normal cell surface morphology. Bar = 10 µm.

Fig. 8. Platinum/carbon surface replica of infected cells treated with 10 µm ionophore in the presence of 100 µM $CaCl_2$. Microvilli have disappeared. Bar = 10 µm.









Disassembly of preformed budding virus structures by Ca^{2+} ions

The data indicated so far that actin filaments composing the submembranous cortical network (Wolosewick and Porter, 1979) are involved in the formation of viral buds at the plasma membrane. As calcium ions are known to influence the morphology of the actin filament network as well as in the contractility of non-muscle cells (reviewed by Adelstein, 1982; Schliwa, 1986), we assumed that these ions might also play a role in the process of virus budding at the plasma membrane. To study the influence of calcium ions, infected cells were treated with the calcium ionophore A23187 (Reed and Lardy, 1972). The ionophore was dissolved in DMSO as a stock solution of 10 mM and added to a Ca^{2+}- and Mg^{2+}-free Hepes buffered salt solution immediately before use. Treatment of infected cells with the ionophore in the presence of Ca^{2+} resulted in a complete redistribution of viral surface antigens within less than 5 min, as was determined by immunofluorescence (not shown here). The minimal concentrations of ionophore and of calcium inducing this effect were 10 µM and 100 µM respectively. Redistribution of the viral surface antigens could not be achieved in the presence of the ionophore alone or in the presence of ionophore and Mg^{2+} or Ba^{2+}.

Replicas prepared from infected cells that had been treated with ionophore and Mg^{2+} showed the normal cell surface morphology of HeLa cells (Fig. 7). In contrast microvilli and budding virus structures were completely absent on cells treated with ionophore in the presence of Ca^{2+} (Figs.8 and 9a). As shown at higher magnification immunogold labeled measles virus hemagglutinin was randomly distributed at the cell surface (Fig. 9b). The gold label is located on the shadowed exoplasmic surface as indicated by the shadow of the gold particles (Fig. 9c).

The data suggested that assembled virus structures at the plasma membrane were disassembled by treatment with ionophore and Ca^{2+}. We were then interested to see if the association of the viral nucleocapsids with the protoplasmic face of the plasma membrane were also altered with this treatment. Therefore we prepared replicas of isolated apical plasma membranes from infected cells according to Rutter et al. (1985). The cells were labeled with anti-measles antibodies and with protein A-gold at the surface prior to the isolation of the membranes, and were then processed for the preparation of replicas. Membranes prepared from cells treated with the ionophore and Mg^{2+} showed the typical attachment of nucleocapsids to the protoplasmic membrane face (Fig. 10a). There was a strict co-localization of these nucleocapsids with the gold label at the external surface (Fig. 10b). In contrast, nucleocapsids were not present on the protoplasmic surface of membranes prepared from ionophore and Ca^{2}-treated cells (Fig. 11a and b). The absence of a shadow around the gold particles in Figs. 10 and 11 (compare with Fig.9c) indicates that the label is located at the non-shadowed exoplasmic surface. The data suggest that virus-specific cell surface antigens become randomly redistributed due to the disruption of their linkage to the underlying nucleocapsids.

Fig. 9. Platinum/carbon surface replica of an infected cell treated as described in Fig. 8 and labeled with antibodies to measles virus hemagglutinin and protein A-gold. (a) Virus structures are absent and (b) hemagglutinin is randomly distributed. (c) Gold label at high magnification. Bar (a) = 5 µm; (b) = 0.5 µm; (c) = 0.1 µm.

Discussion

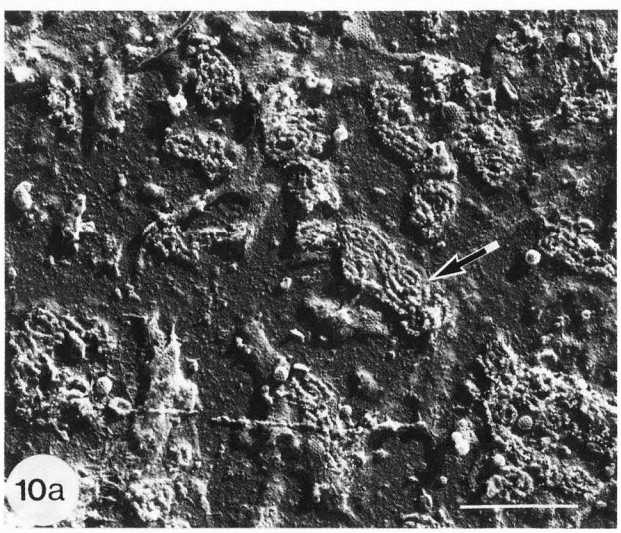
Here we have shown that the combination of the replica method with immunogold-labeling significantly contributes to the understanding of the budding process of measles virus at the plasma membrane. It can be seen that the replica method itself is of advantage to demonstrate three-dimensional alterations of the cell morphology at high resolution in TEM. Cells made into replicas were exclusively grown on glass coverslips which considerably facilitated the handling of the probes because there was no need for cultivation of the cells on special substrates, such as carbon coated formvar-grids. In addition the individual preparation steps could be monitored in parallel by immunofluorescence as well as by SEM (Hohenberg et al., 1986). The electron-opaque gold particles allowed an easy identification of viral structures on platinum/carbon shadowed cell surfaces, cytoskeletons and membranes. It may be assumed that cleaning of surface replicas with chromic acid may lead to a loss of gold particles from the replicas. However, cleaned surface replicas showed the same labeling pattern of hemagglutinin as was found on isolated apical plasma membranes which were not treated with chromic acid. Furthermore, preliminary studies showed no differences in the localization of gold particles on shadowed cell surfaces prior to and after treatment with chromic acid. These data seem to confirm the reliability of the immunogold-replica technique.

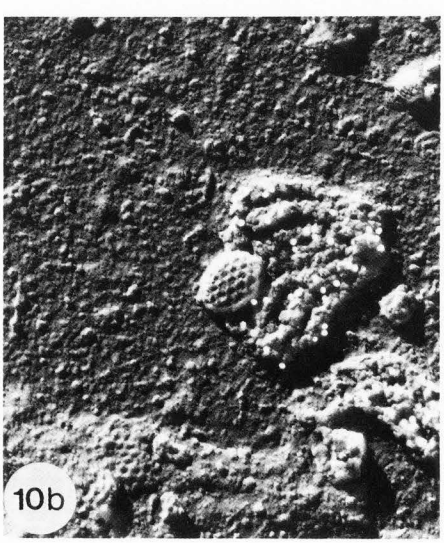
By use of these techniques we studied (1) the process of measles virus budding at the cell surface, (2) the association of viral structures with the cytoskeleton, and (3) the relationship between viral

antigens at the cell surface and viral structures located on the protoplasmic face of the plasma membrane.

In order to follow the sequence of budding at the plasma membrane, we were forced to treat infected cells with drugs that were able to influence the cell surface morphology. By use of phenothiazines the sequence of virus budding could be dissected, showing that virus specific strands and stub-like protrusions are transitory stages in this process. Furthermore, the results also suggest that budding and the formation of cellular microvilli seem to have similar underlying mechanisms. The formation of both structures seems to be dependent on the function of the microfilament system. Although phenothiazines are known to inhibit Ca^{2+}-calmodulin-dependent enzyme activities (Weiss et al., 1980), our results do not give direct evidence for a participation of calmodulin in virus budding, being aware of side effects of these drugs on cellular metabolism (Corps et al., 1982).

The studies done on cytoskeletons of infected cells confirmed our assumption that budding takes place in close interaction with the cytoskeleton. These findings also explain results obtained by others with immunofluorescence and the application of cytochalasin B (Sundqvist and Ehrnst, 1976; Tyrell and Ehrnst, 1979). Stereo replicas clearly showed that viral structures were associated with cytoskeletal filaments composing the outer parts of the cytoskeletons. These viral structures retained their normal morphology despite the use of a rather high detergent concentration and a relatively long extraction time. Usually lower concentrations of nonionic detergents (0.1-0.5%) are used to prepare cytoskeletons, and extraction under these conditions was found to be complete within an exposure time of less than 1 min (Schliwa et al., 1981). Decoration of actin filaments with heavy meromyosin was not as clearly visible on our rotary shadowed cytoskeletons as was shown with filaments of quick-frozen and deep-etched intestinal brush border microvilli (Hirokawa et al., 1982). Probably fixation,





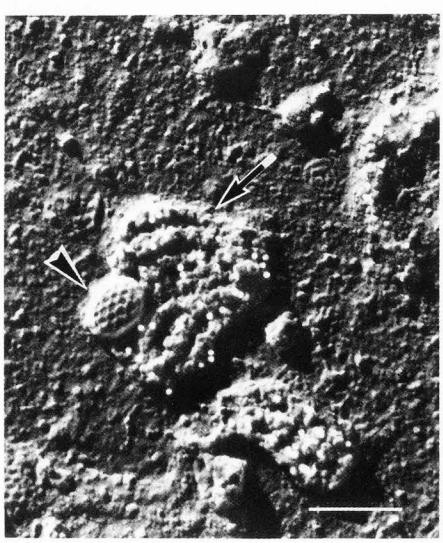


Fig. 10. Platinum/carbon replicas of isolated apical plasma membranes prepared from infected cells. (a) Membranes isolated from ionophore and $MgCl_2$ treated cells show the typical attachment of nucleocapsids (➞) to the protoplasmic membrane face. (b) Stereo images show that the localization of nucleocapsids (➞) corresponds to that of immunogold-labeled virus antigens at the outer cell surface; clathrin basket (►). Bar (a) = 1 µm; (b) = 0.25 µm.

dehydration and critical point drying are detrimental for the preservation of the fine structural morphology. For instance, freeze drying seems to provide better resolution of individual actin filaments relative to critical point drying (Hartwig and Shevlin, 1986). However, ultrathin sectioning of extracted cells, decorated with heavy meromyosin gave evidence for the binding of actin filaments to nucleocapsids in budding virus structures (Bohn et al., 1986). The orientation of actin filaments within these structures suggests that budding is connected with a vectorial growth of actin filaments.

The method developed for the preparation of apical plasma membranes enabled us to localize viral nucleocapsids on the protoplasmic membrane face and to study their relationship to labeled viral antigens at the cell surface. The sideness of labeling relative to shadowing can easily be identified with stereo views and due to the presence or absence of a shadow around the gold particles. The structures designated as nucleocapsids show the typical length ($\sim$ 1 µm) and diameter ($\sim$ 30 nm) of measles virus nucleocapsids (Dubois-Dalcq and Barbosa, 1973), they are exclusively localized beneath labeled viral glycoproteins at the cell surface, and they can be labeled with antibodies against nucleocapsid associated proteins (Rutter et al., 1986). However, they did not show the typical helical arrangement of subunits demonstrated by others on isolated nucleocapsids which were quick-frozen, deep-etched and rotary shadowed (Dubois-Dalcq et al., 1984). It has to be determined if this method, or alternatively freeze drying, can provide more fine structural details on membrane associated nucleocapsids.

We could demonstrate that the association of nucleocapsids with the plasma membrane can be affected by increasing the intracellular calcium concentration. This we did using the calcium ionophore A23187, which mediates the electroneutral exchange of divalent cations for protons (Pressman et al., 1967) and has been used to demonstrate Ca^{2+}-dependent processes in intact cells (reviewed by Campbell, 1983). Virus structures at the plasma membrane are disassembled only in the presence of Ca^{2+} but not of Mg^{2+}, speaking for a specific effect of Ca^{2+} ions. The results obtained with isolated plasma membranes point to the fact that Ca^{2+} ions disrupt the linkage between nucleocapsids and the viral envelope, and thereby induce a random distribution of viral envelope proteins. Although we have no clear evidence for the way Ca^{2+} leads to virus-disassembly, our data suggest that the morphogenesis of measles virus at the plasma membrane may underlie the control of Ca^{2+} ions.

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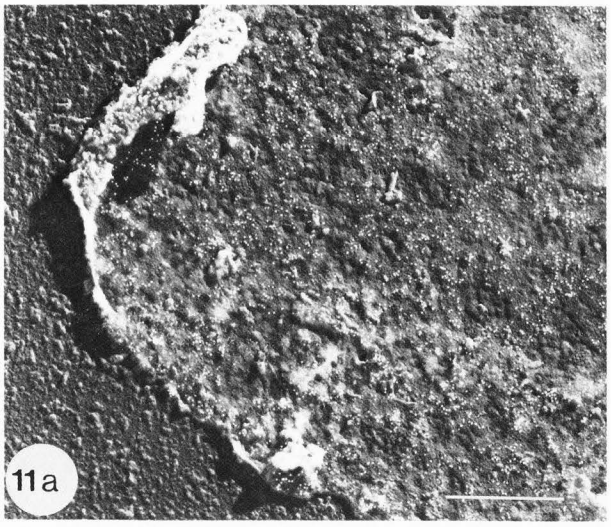

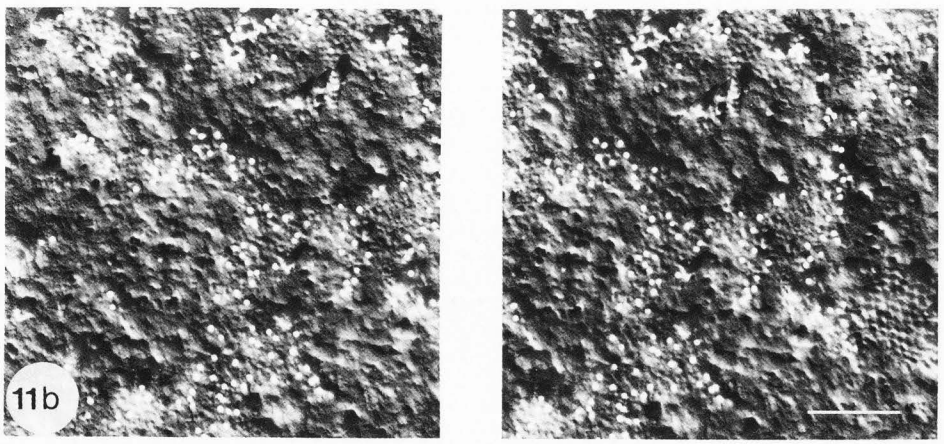

Fig. 11. Platinum/carbon replicas of isolated apical plasma membranes prepared from ionophore and $CaCl_2$ treated infected cells. Stereo views (b) show that nucleocapsids are not visible on the protoplasmic membrane face. Whereas the immunogold-labeled virus-specific antigens on the outer cell surface are still detectable. Bar (a) = 1 µm; (b) = 0.25 µm.

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Discussion with Reviewers

E.J. Basgall: Have you compared the results obtained with NP40 to any other non-ionic detergents, such as TX-100, in order to determine if a selective extraction or re-localization of viral components may be occurring?

Reviewer III: Will the putative virus particles be dissolved in higher concentration of detergent or after prolonged treatment?

Authors: No difference was found between extraction with NP40 or Triton X-100. No other non-ionic detergents were tested. Even prolonged incubation with the lysis buffer (up to 20 min) did not disrupt cytoskeleton bound virus particles. However, we did not use higher concentrations of detergent than 1%. The extraction procedure did not lead to a general contamination of uninfected cells with virus structures as determined by immunofluorescence. Furthermore, viral glycoproteins showed the same distribution pattern whether immuno-labeling was done prior to or after extraction. These data speak against a random relocalization of viral components during extraction.

Reviewer III: Figs. 3 and 4 show that delipidisation was not complete. This would explain the preservation of some infectivity after NP40.

Authors: We have not done any experiments to determine the extent of delipidisation, but an incomplete delipidisation indeed may be responsible for the preservation of infectivity. Preservation of infectivity after NP40 treatment is a characteristic property of cell bound virus. Supernatant infectivity is completely inactivated. Possibly the binding to the cytoskeleton stabilizes the virus structures and thereby preserves the infectivity.

E.J. Basgall: You used meromyosin to coat actin filaments for identification. Is there a reason that anti-actin:PAG was not utilized in this study?
Authors: Binding of heavy meromyosin (HMM) to actin filaments represents a functional interaction, and with this method we hoped to identify the filament polarity on shadowed cytoskeletons. In addition this procedure can be combined with immunogold-labeling of virus structures. Studies are in progress to improve the preservation of fine structural details of decorated filaments and to combine the decoration with immunogold-labeling.

Reviewer III: Have you considered extracting cells grown on grids followed by light rotary shadowing and stabilisation by light rotary carboning? This would obviate the need of replication, which could cause problems and some distortions caused by floating and cleaning of the replicas.
Authors: We only used coverslip-cultures because this system is easy to handle and comparative immunocytochemical and ultrastructural studies can be done on cell surfaces, cytoskeletons, isolated membranes, and freeze-fractured cells by use of immunofluorescence, SEM and TEM. As previously shown (Hohenberg et al., 1986) special equipment and handling procedures developed in our laboratory minimize mechanical distortions.

E.J. Basgall: When you treat with A23187 and Ca^{++} there appears to be a marked effect upon the cell morphology, notably a pulling away from adjacent cells. Could this be due to a toxicological effect or an artefact of the drying process?
Authors: Most probably this is due to the toxic effect of calcium in the presence of the ionophore. This phenomenon can also be detected in immunofluorescence and is not present in control preparations. Treatment with calcium and ionophore induces severe disruptions of the cytoskeleton, which seems to induce retraction of the cell.

Reviewer III: At higher concentrations used here the effects become non-specific. A23187 acts like a detergent as 10 µM.
Authors: We have no evidence for a detergent effect of the ionophore, even at a concentration of 20 µM. The effects described are clearly dependent on the presence of calcium and cannot be induced by the ionophore alone.

Reviewer III: Fig. 6 shows a hairy control cell. How does it compare with Fig. 1? It would be rather difficult to prepare apical membranes from such a cell.
Authors: The cells normally are polymorph. The presence of numerous microvilli does not impair the isolation of apical plasma membranes as described by Rutter et al. (1985).

Reviewer III: How were the replicas of the apical plasma membranes cleaned to prevent loss of gold label?
Authors: The protoplasmic face of the membrane was shadowed. The gold label is located at the exoplasmic membrane face. After shadowing the membranes were detached from the glass, transferred onto distilled water and mounted on grids. They were not cleaned with chromic acid.

Reviewer III: Fixation of cells before immunolabelling is usually a compromise between preservation of antigenicity and structural feature. Have you carried out systematic studies comparing the effect of different fixatives on the monoclonal and polyclonal antibody labelling?
Authors: Combinations of different glutaraldehyde and paraformaldehyde concentrations were tested. We have chosen the lowest aldehyde concentration resulting in a reasonable preservation of cell morphology without significantly affecting the antigens detected by our monoclonal and polyclonal antibodies. The aldehyde mixture we selected resulted in a very low background label as previously shown (Mannweiler et al., 1982). Higher concentrations of glutaraldehyde significantly increased the background label, but did not improve the ultrastructure.

E.J. Basgall: Have you tried any other preparative drying techniques such as drying with CO_2 rather than Freon 13?
Authors: We did not perform any other drying procedures.