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Release of Progeny Virus from Cells Infected with Simian Rotavirus SA11

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SUMMARY

Analysis of cells infected with simian rotavirus SA11 at late times of infection indicated that the particles were associated with membranes and the cytoskeleton. Although a large amount of cellular and non-structural viral proteins were released at these times, probably by cellular lysis, only virus with an outer layer was found outside the cells, while virus without an outer layer remained associated with the cells, probably with membranes and the cytoskeleton. Inhibition of glycosylation by tunicamycin did not abolish cell lysis but inhibited the liberation of particles and the non-glycosylated precursors of the structural and non-structural viral glycoproteins. These results indicate that immature virus was tightly associated with the structural matrix of the cell.

INTRODUCTION

The genome of SA11 rotavirus consists of 11 segments of dsRNA (Malherbe & Strickland-Cholmley, 1967) which is packed in a core containing two protein classes, VP1 and VP2 (Patrick et al., 1982). This core is surrounded by another protein, VP6, which forms the inner layer (Patrick et al., 1982). These particles are found in partially purified preparations and are usually called 'heavy particles' (Elias, 1977). The virions, also called 'light particles' according to their buoyant density (Rodger et al., 1977), contain an outer layer formed by at least two protein classes, VP3 and VP7 (Espejo et al., 1981). VP7 is a glycoprotein containing N-linked oligosaccharides of the high-mannose type (Arias et al., 1980).

Rotavirus morphogenesis seems to be unique. Particles morphologically similar to virus devoid of the outer layer assemble in cytoplasmic inclusions (viroplasms) at the periphery of the nucleus and bud through the membranes of the rough endoplasmic reticulum (Altenburg et al., 1980; Chasey, 1977; McNulty et al., 1976; Petrie et al., 1982). The envelope acquired in this process appears to be lost as the particles move towards the interior of the endoplasmic reticulum cisternae and, instead of the membrane, a thin layer of proteins is found in virions. If the enveloped particles are precursors of the virions, as is generally assumed, the process by which the membrane is transformed into a thin layer of protein, or replaced by it, is unique and its mechanism one of the most interesting problems of rotavirus morphogenesis.

Studies of the synthesis of VP7 and of the non-structural glycoprotein NCVP5 (NC stands for non-capsid) in vitro and in vivo (Ericson et al., 1983), indicates that they are synthesized in the endoplasmic reticulum membranes in which they are inserted but behave differently in later steps. VP7 seems to be inserted totally into the microsomal lumen and might be assembled into the outer protein shell of the virus particles as the subviral particles bud through the rough endoplasmic reticulum membrane (Petrie et al., 1982). NCVP5, on the other hand, behaves like a transmembrane protein (Ericson et al., 1983), and therefore would be present in both the budding and enveloped subviral particles but would later be lost along with the lipid bilayer.

The inhibition of glycosylation of VP7 and NCVP5 (Petrie et al., 1983) by tunicamycin (TM) blocks the formation of the outer layer but neither the insertion of the glycoproteins into the membrane nor the budding of the subviral particles. Therefore, the overall effect is the accumulation of membrane-enveloped particles in the lumen of the endoplasmic reticulum (Petrie et al., 1983). Since TM has the same effect in a viable variant of SA11 which contains
non-glycosylated VP7, the glycosylation of NCVP5 seems to be a stage required for completion of morphogenesis in SA11 (Petrie et al., 1983).

From electron microscopy of the thin sections of infected cells, it has been suggested that the progeny virus in the lumen of the endoplasmic reticulum is released at late times after cell lysis (Altenburg et al., 1980). In this article, we describe the release of viral particles and proteins both in normal infections and in the presence of tunicamycin, which was studied in an attempt to understand the late steps in the process of rotavirus replication.

**METHODS**

*Cells and virus.* MA104 cells were grown as monolayers in 25 cm² disposable flasks in Eagle's MEM, supplemented with 10% foetal calf serum, penicillin (100 μg/ml), and gentamicin (160 μg/ml).

Simian rotavirus (SA11), obtained originally from H. H. Malherbe (University of Texas, San Antonio, Texas, U.S.A.) was plaque-purified three times as previously described (Estes et al., 1982). Since the SA11 structural glycoprotein (VP7) exhibits heterogeneity, we used a clone coding for the 38000 molecular weight glycosylated VP7 (Estes et al., 1982), designated 2-82.

Radioactively labelled infected cells. MA104 confluent monolayers were washed twice with phosphate-buffered saline (PBS) and infected with SA11 virus in the presence of trypsin (10 μg/ml). In experiments involving tunicamycin (a gift from P. L. Hamill of Lilly Research Laboratories), the drug was added to the cell during the adsorption period at a concentration of 5 μg/ml. This concentration was used in all subsequent steps. The virus was allowed to adsorb to the cells for 1 h at 37 °C. The medium was then replaced by MEM which had a lower than normal concentration of L-methionine (0.3 mg/l) and the cells were labelled either immediately or 4 h later with 100 μCi L-[35S]methionine (> 500 Ci/mmol, New England Nuclear).

At different times after infection, medium was removed from the monolayer and the cells were resuspended in TSM buffer (0.01 M-Tris–HCl, 0.15 M-NaCl, 0.001 M-MgCl₂·6H₂O, pH 8.2) and extracted with an equal volume of trifluorotrichloroethane (Freon). Viral particles present either in the aqueous phase or in the culture medium were analysed as described below. In some experiments, cells were pulse-labelled for 30 min at 4 h post-infection, washed three times with warmed MEM, and further incubated for the desired chase period.

**Fractionation of membranes.** The monolayer was washed once with cold, calcium- and magnesium-free PBS and once with ET buffer (0.02 M-Tris–HCl, 0.001 M-EDTA, pH 8.2). The washed cells were suspended in 2 ml ET buffer containing 1 mM-phenylmethylsulphonyl fluoride (PMSF; Sigma). The cells in the monolayer were then allowed to detach and the cell suspension was homogenized by 25 strokes of a Dounce homogenizer. The cell homogenate was subsequently layered on a discontinuous gradient consisting of 6 ml 60% sucrose (w/v in ET buffer) and 8 ml of 10% (w/v) sucrose and was centrifuged in a Sorvall HB4 swinging bucket rotor at 12000 r.p.m. for 60 min at 5 °C. Fractions were then collected and an aliquot was assayed for radioactivity after precipitation with 10% TCA. Those fractions containing the membranous material were pooled and extracted with an equal volume of Freon and the aqueous phase was analysed for the presence of viral particles as described below. The last fractions, corresponding to the meniscus of the gradient, were pooled and analysed for the presence of viral particles.

Detergent extraction. Medium was removed from the monolayer and the cells were washed once with cold, calcium- and magnesium-free PBS and once with extraction buffer (EB: 10 mM-Tris–HCl, pH 7.4, 50 mM-NaCl, 2.5 mM-MgCl₂, 300 mM-sucrose, 1 mM-PMSF) as previously described (Ben Ze'ev et al., 1979). Extraction buffer containing 1% Triton X-100 was then added and, after 5 min on ice, the supernatant was removed and the cellular material remaining attached to the plastic was suspended in EB containing 1% Triton X-100. The soluble fraction and the insoluble Triton X-100 fraction were treated with Freon and subsequently analysed for the presence of viral particles as described below.

To test the efficiency of the extraction procedure, cells were labelled with 32 μCi [32P]orthophosphate (10 mCi/ml, New England Nuclear) for 24 h and then infected with SA11. After 15 h of infection, the cells were washed and extracted with Triton X-100 as described above. The [32P]-labelled phospholipids were isolated from the soluble and skeletal fractions by the chloroform-methanol extraction procedure (Kates et al., 1961) and the radioactivity was determined.

**Analysis of viral particles.** Viral particles present in the different samples were centrifuged for 1 h at 40000 r.p.m. in a Beckman SW55 rotor. After centrifugation, the pellet material was resuspended in 200 μl TSM buffer and then layered onto preformed CsCl gradients (1.3 to 1.4 g/ml in TM buffer: 0.01 M-Tris–HCl, 0.001 M-MgCl₂·6H₂O, pH 8.2). The gradients were centrifuged at 36000 r.p.m. in a Beckman SW40 rotor for 3 h and fractionated by bottom displacement. Samples of each fraction were TCA-precipitated onto Whatman filters and radioactivity was counted.

**Haemagglutination.** Haemagglutination tests were carried out using microtitre equipment. The diluent used throughout was PBS pH 7.8, containing 0.5% bovine serum albumin. Doubling dilutions of samples were incubated at room temperature for 1 h with equal volumes of 0.5% human group O erythrocyte suspension in PBS.
Titres are expressed as the reciprocal of the last dilution that showed haemagglutination per cell.

Polyacrylamide gel electrophoresis of polypeptides. Analysis of the polypeptide composition of the different samples was carried out in 12% polyacrylamide gels, using the discontinuous Tris–glycine buffer system (Laemmli, 1970), containing 0.5 M-urea. Samples were boiled for 2 min with Laemmli's sample buffer containing 0.5 M-urea. Polypeptides were stacked at 10 mA/gel. After the separation run, gels were processed for fluorography by the method of Bonner & Laskey (1974).

Viable cell count. Attached cells were resuspended in 0.025% trypsin and added to the original growth medium containing detached cells. Trypan blue (Gibco) dissolved in PBS was added to a final concentration of 0.08%. After 5 min a total cell count and a stained cell count were done using a haemocytometer.

RESULTS

Infection of MA104 cells with SA11 results in extensive c.p.e. (Estes et al., 1979). The main visible changes are cytoplasmic vacuolation and the appearance of small eosinophilic intracytoplasmic inclusions. Cytopathic effect is maximal at 24 h post-infection with 100% of the cells being detached from the monolayer. It has been assumed that infected cells lyse, but it has also been reported that only 20% of the infectious virus is released into the medium, while the rest remains associated with the cells at late times after infection (Estes et al., 1979). In preliminary experiments the release of virus was estimated measuring the release of viral haemagglutinin.

Haemagglutinating activity was first detected in the culture medium 13 h post-infection and reached its maximum at 15 h post-infection with a titre of $8 \times 10^{-3}$ haemagglutinating units per cell. This amount corresponded to at most one-third of the total haemagglutinin produced, since twice that amount could be released from the pelleted cells after Freon treatment.

It has been shown by several workers that incomplete, single-shelled particles can easily be separated from double-shelled complete particles by CsCl gradient centrifugation. To study the release of the different viral particles, infected cells were labelled with $[^{35}\text{S}]$methionine and the viral particles present in the culture medium and those remaining associated with the cells, but released after Freon treatment, were analysed in CsCl gradients. Fig. 1 shows that only double-shelled particles were released into the culture medium whereas single-shelled and also some double-shelled particles remained associated with the cells and were released only after Freon treatment. However, the particles without an outer layer which were found associated with the cells may have been derived from membrane-enveloped particles, which have previously been described in infected cells (Petrie et al., 1981, 1983), as the result of disintegration of the membrane during treatment with Freon.

Our results which showed a specific release of mature virus were inconsistent with the assumption that there is extensive cytolysis during SA11 infection. However, further experiments, in which the release of cellular proteins (labelled prior to infection), the penetration of trypan blue, and the release of total proteins labelled during infection (Table 1)

Table 1. Release of proteins after infection with SA11

<table>
<thead>
<tr>
<th>Release of cellular proteins* (24 h post-infection)</th>
<th>Uninfected (%)</th>
<th>Infected (%)</th>
<th>Infected + tunicamycin (%)</th>
<th>Uninfected + tunicamycin (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staining with trypan blue† (18 h post-infection)</td>
<td>7</td>
<td>95</td>
<td>ND§</td>
<td>ND</td>
</tr>
<tr>
<td>Release of viral proteins‡ (18 h post-infection)</td>
<td>ND</td>
<td>29</td>
<td>44</td>
<td>ND</td>
</tr>
</tbody>
</table>

* MA104 cells were labelled with $50 \mu \text{Ci/ml}[^{35}\text{S}]$methionine for 2 h prior to infection. At 24 h post-infection, an aliquot of the medium was precipitated by TCA onto Whatman filters and counted. To measure the total radioactivity incorporated, cells were lysed with 1% SDS and the acid-precipitable radioactivity was determined. Results are presented as the percentage of acid-precipitable radioactivity present in the medium. Control cultures were mock-infected with PBS.
† Described under Methods.
‡ SA11-infected cells were pulse-labelled for 30 min at 4 h post-infection. At 18 h post-infection, the medium was removed and the cells were suspended in TE buffer and homogenized. An aliquot of the medium and cells were counted after TCA precipitation. Results are presented as the percentage of acid-precipitable radioactivity present in the medium.
§ ND. Not done.
were measured, showed that there was indeed a drastic alteration in the permeability of the plasma membrane of the infected cells, resulting in extensive release of cellular and viral proteins. Although there was cell lysis, all single-shelled particles and a great proportion of double-shelled particles remained cell-associated, suggesting that there was an interaction with some structures within the cells. Pulse-labelled, SA11-infected cells were homogenized after an 18 h chase period and centrifuged on a sucrose gradient to study the possible association of viral particles with the cellular membranes. It was found that most of the label was distributed into two main components, a 'membrane' fraction that sedimented on top of the 60% sucrose layer and a 'soluble' fraction on top of the gradient where free virus should remain (Fig. 2a). When both fractions were treated with Freon to release associated particles and subsequently were analysed in CsCl gradients, viral particles were only observed in the fast-sedimenting 'membrane' fraction indicating that there were no free intracellular particles (Fig. 2b). However, in experiments in which viral particles were solubilized by non-ionic detergents not all
Fig. 2. Association of viral particles with membranes. (a) Fractionation of membranes. SA11-infected cells were pulse-labelled with $^{35}$S-methionine for 30 min at 4 h post-infection. At 18 h post-infection, the medium was removed and cells were suspended in ET buffer and homogenized. The homogenate was then layered on a discontinuous sucrose gradient and centrifuged. Fractions were collected and counted after TCA precipitation. (b) Fractions containing the membranous material from the gradient in (a) were pooled and extracted with an equal volume of Freon and the aqueous phase was analysed in a CsCl gradient, as described in Methods. $\bullet$, Radioactivity; $\bigcirc$, density.

Fig. 3. Association of viral particles with the cell cytoskeleton. SA11-infected cells were labelled with $^{35}$S-methionine at 4 h post-infection. At 15 h post-infection, the medium was removed and the cells were washed and extracted with 1% Triton X-100 as described in Methods. The soluble fraction and the insoluble fraction were analysed in CsCl gradients, after Freon treatment. $\bullet$, Radioactivity; $\bigcirc$, density. (a) Medium; (b) Triton X-100-soluble fraction; (c) Triton X-100-insoluble fraction.

the intracellular particles were liberated (not shown), suggesting that particles were not exclusively associated with membranes. Viral particles might also be associated with the cell cytoskeleton since the cytoskeletal framework remains after the lipids and soluble proteins have been extracted from cells by a non-ionic detergent (Ben Ze’ev et al., 1979). In order to test this possible association, SA11-infected cells labelled with $^{35}$S-methionine were gently extracted with 1% Triton X-100 to leave behind the cytoskeleton. Analysis of viral particles remaining in the cytoskeleton, but later released by Freon treatment, and of those present in the Triton X-100-
soluble fraction showed that particles with an outer layer were preferentially released into both the medium and the Triton X-100-soluble fraction, whereas most particles without an outer layer remained associated with the cytoskeleton (Fig. 3). The association of particles with the cytoskeleton was very stable and was not broken by homogenization or sonication (results not shown).

The protein composition of these particles after being released by Freon treatment and that previously reported for purified single- and double-shelled virus (Fig. 4) were similar and eliminated the possibility that other proteins occurred in the particles remaining associated with the cytoskeleton.

It has been demonstrated in a variety of systems that tunicamycin interferes with the glycosylation of glycoproteins without affecting other cellular processes (Gibson et al., 1978; Schwarz et al., 1976; Takatsuki et al., 1971). When cells were infected in the presence of tunicamycin, it was found that no viral particles were released into the culture medium and that all the particles that remained cell-associated appeared single-shelled after Freon treatment.
Rotavirus progeny release

![Graph](image)

Fig. 5. CsCl density gradient analysis of SA11 viral particles released and cell-associated in cell cultures infected in the presence of tunicamycin. Tunicamycin was added to the cells during the adsorption period (2.5 μg/ml) and this concentration was maintained throughout the infection period. [35S]Metionine was added after the adsorption period. At 24 h post-infection, the culture was harvested and analysed as described in Methods. △, 35S released into the medium; ●, 35S cell-associated; ○, density corresponding to the gradient containing cell-associated 35S.

(Fig. 5). All the particles synthesized in the presence of tunicamycin were apparently associated with the cytoskeleton since they were released into the Triton X-100-soluble fraction (data not shown). To find whether there was also a preferential distribution of viral proteins in the medium or in the Triton X-100-soluble and -insoluble fractions similar to that found with viral particles, we analysed the protein composition of the different fractions obtained in the presence and absence of tunicamycin. Interestingly, we found that in the absence of glycosylation, the non-glycosylated precursors of VP7 and NCVP5 were not released into the culture medium but remained cell-associated, in spite of the fact that there was extensive cell lysis such as that found in normal infection (Fig. 6). Unexpectedly NCVP5, which has been shown to be a transmembrane protein (Ericson et al., 1983), was not fully solubilized by Triton X-100 and a proportion of it remained associated with the cytoskeleton. This association was not due to inefficient extraction with Triton X-100 because 80 to 90% of the 32P-labelled phospholipids could be extracted from infected and uninfected cells in control experiments.

DISCUSSION

From observations of SA11-infected cells by thin section electron microscopy, it has been suggested that, at times late after infection, cells lyse releasing progeny virus into the culture medium (Altenburg et al., 1980; Chasey, 1977; McNulty et al., 1976; Petrie et al., 1982). Our results support this suggestion. However, although a large amount of cellular proteins and both structural and non-structural viral proteins were released, only complete particles were detected in the culture medium, whereas all single-shelled particles and a portion of the complete particles remained associated with either membranes or cytoskeleton.

The observation that the non-structural glycoprotein, NCVP5, was released into the culture medium is especially interesting in view of the finding that it is a transmembrane protein (Ericson et al., 1983). Maturation of rotavirus is believed to occur after the particles bud through the rough endoplasmic reticulum, acquiring a membrane envelope in the process. The function of this envelope is of particular interest since it appears to be transient and is not required for infectivity (Petrie et al., 1981). It is possible that NCVP5, as well as other viral proteins, is present in this envelope and, when transformation into the outer protein shell occurs, it is lost along the lipid bilayer and released into the culture medium after cell lysis.
Since tunicamycin is generally accepted to affect only glycosylation without having other effects on protein synthesis (Takatsuki et al., 1971), we used it as a selective inhibitor in our studies. Inhibition of glycosylation with tunicamycin yielded viral particles without an outer layer as previously described (Petrie et al., 1983), but all particles remained cell-associated although cell lysis was also observed under these conditions. These particles, detected as single-shelled virus, may have been derived from membrane-enveloped particles, previously shown to accumulate in tunicamycin-treated cells (Petrie et al., 1983; Soler et al., 1982) due to the destruction of the envelope by the Freon treatment employed to release the associated viral particles. In the presence of tunicamycin, the non-glycosylated precursors of VP7 and NCVP5 were not found in the culture medium probably because they remained anchored to the cell membrane and cytoskeleton.

Our studies have shown an association of viral particles and proteins with the cell cytoskeleton. We have found that particles mainly without an outer layer remained associated with the cytoskeletal framework. These particles may have been derived as described above. The cytoskeleton may provide a means of transport of viral proteins and particles to the rough endoplasmic reticulum and then may act as a stabilizing element at the assembly site and in
Rotavirus progeny release

newly budded virions. The membrane-enveloped particles may be linked to the cytoskeleton through viral proteins present in their membranes; this association may be lost when the viral membrane envelope is transformed into the outer protein shell.

A role for the cytoskeleton in the growth and assembly of other viruses has been proposed. For example, reovirus infection produces a major disruption and reorganization of vimentin filaments; it has been suggested that these filaments may participate in the formation of unique viral structure(s) that function(s) as sites of viral replication and assembly (Sharpe et al., 1982). In Rauscher murine leukaemia virus, a rapid and specific association of the precursor polyprotein Pr65Gag with cytoskeletal elements in infected mouse fibroblasts is observed and it has been proposed that this association plays some role in virus assembly (Edbauer & Naso, 1983). Infection of tissue culture cell with canine distemper virus causes a total reorganization of all the cytoskeletal structures with the most notable changes in the microtubules and intermediate filaments (Howard et al., 1983).

Since colchicine or cytochalasin treatment of rotavirus-infected cells does not reduce viral yields (C. Soler & M. A. Lorofio, personal communication), it is unlikely that microtubules or microfilaments play a role in viral growth. A possible essential role of intermediate filaments remains to be tested.

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