Localization of Rotavirus Antigens in Infected Cells by Ultrastructural Immunocytochemistry

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SUMMARY

Virus structural antigens were localized within a line of monkey kidney (MA104) cells infected with the simian rotavirus SA11 using electron microscopic immunoperoxidase techniques. When hyperimmune guinea-pig anti-SA11 serum was used, virus particles, membranes of virus-associated endoplasmic reticulum, and viroplasmic inclusions were most heavily labelled. A general cytoplasmic reaction (ribosomes, intracytoplasmic membranes, etc.) with anti-SA11 serum was also observed, but nuclei were unstained. In addition, several other virus-induced structures were found to contain rotavirus proteins, including convoluted smooth membrane within the endoplasmic reticulum, aberrant virus-like particles, and 15 to 20 nm diam. cytoplasmic tubules. Monospecific antiserum to VP7 (outer capsid glycoprotein, mol. wt. 38000) reacted strongly with virus particles and the virus-associated endoplasmic reticulum, but reacted poorly with viroplasmic inclusions. The nucleus and general cytoplasm were unstained with anti-VP7. In contrast, monospecific antisera to VP2 and VP6 (inner capsid proteins, mol. wt. 94000 and 41000 respectively) reacted very strongly with viroplasmic inclusions. Virus particles, endoplasmic reticulum and cytoplasmic ribosomes were also labelled with these sera. These results indicate that rotavirus inner capsid proteins are synthesized throughout the cytoplasm and become concentrated in viroplasmic inclusions, while the outer capsid glycoprotein is synthesized primarily on ribosomes of the rough endoplasmic reticulum. Thus, the outer capsid layer appears to be acquired during virus budding into cisternae of the endoplasmic reticulum.

INTRODUCTION

Rotaviruses, originally identified as veterinary pathogens (Mebus et al., 1969), are now recognized as a major cause of gastroenteritis in young children and a variety of mammalian and avian species (Flewett & Woode, 1978; McNulty et al., 1979). A comparison of the morphogenesis of the simian rotavirus SA11 (Lecatsas, 1972; Altenburg et al., 1980) with electron microscopic observations of rotavirus replication in tissues from infected animals and in cultured cells (Adams & Kraft, 1967; Banfield et al., 1968; Lecatsas, 1972; Stair et al., 1973; Holmes et al., 1975; Hall et al., 1976; McNulty et al., 1976; Chasey, 1977; Saif et al., 1978; Pearson & McNulty, 1979) demonstrated that SA11 is a useful representative model for the study of rotavirus infections. Our previous studies (Altenburg et al., 1980) show that areas of dense granular material (viroplasm) begin to accumulate in the perinuclear region of the cytoplasm 8 h after infection. Virus particles assemble near the periphery of these inclusions and subsequently enter into cisternae of the rough endoplasmic reticulum by a budding process. Many particles become enveloped during budding, but the envelope appears to be easily lost since viruses located near the centres of large cisternae usually lack it. The envelope is not required for infectivity (Elias, 1977; Estes et al., 1979b; Petrie et al., 1981) and its function

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remains unknown. Rotavirus particles have never been noted to bud through the plasma membrane, but rather enter the culture medium following lysis of infected cells.

SA11 morphogenesis also exhibits several features not yet reported for other rotavirus strains. In 1 to 5% of SA11-infected cells, bundles of 15 to 20 nm diam. tubular structures (usually surrounded by an electron lucent space) can be found within the cytoplasm and/or the nucleus (Lecatsas, 1972; Altenburg et al., 1980). In addition, virus particles are sometimes found within the intercristal space of host cell mitochondria (Altenburg et al., 1980). The larger nuclear tubules observed in cells infected with murine (EDIM, epizootic diarrhoea of infant mice) and porcine rotaviruses (Banfield et al., 1968; McNulty, 1978) have not been reported in SA11 infections.

Rotavirus antigens have been localized within infected cells by light microscopy using both immunofluorescence (McNulty et al., 1979) and immunoperoxidase techniques (Graham & Estes, 1979; Altenburg et al., 1980). Although virus antigens can be detected as discrete cytoplasmic inclusions by these techniques, it is not possible to distinguish viroplasms from virus-filled cisternae. Furthermore, the smaller and less frequently observed structures cannot be resolved by light microscopy. Although the major features of rotavirus morphogenesis have been well-established, little is known at the molecular level about the assembly of rotavirus proteins into virions. Preliminary studies on the ultrastructural localization of calf (Chasey, 1980) and SA11 (Altenburg et al., 1979) rotavirus antigens, using peroxidase as the immunological marker, have shown a generalized cytoplasmic distribution of virus proteins in cells undergoing lysis with heavy labelling of virus particles, viroplasmic inclusions and rough endoplasmic reticulum. However, these studies used broadly reacting antisera and examined only a few cells. In the present study, we confirm and extend these results using hyperimmune antiserum to SA11 rotavirus and monospecific antisera against (i) the major glycoprotein VP7 (mol. wt. 38 000) from the outer shell, (ii) the major inner capsid protein VP6 (mol. wt. 41 000), and (iii) the second most abundant inner capsid protein VP2 (mol. wt. 94 000).

METHODS

Cells and virus. Stocks of simian rotavirus SA11 were grown at low multiplicity in cultures of foetal rhesus monkey kidney cells (MA104; Microbiological Associates, Bethesda, Md., U.S.A.) in Eagle’s minimal essential medium (MEM) without foetal bovine serum (FBS) as previously described (Estes et al., 1979). Stocks were titred by plaque assay in MA104 cells under an agar overlay supplemented with pancreatin and DEAE-dextran (Smith et al., 1979).

Antisera. Hyperimmune antiserum to SA11 was prepared in guinea-pigs using purified double- and single-shelled SA11 particles (Mason et al., 1980) as previously described (Estes et al., 1979b). This antiserum has been shown to immunoprecipitate most of the SA11 structural proteins (i.e. VP2 to VP9 but not VP1) from infected cell lysates, whereas preimmune serum from guinea-pigs did not (Mason et al., 1980; Ericson et al., 1982). Immunological specificity was confirmed in the immunocytochemical experiments by absolving the antiserum with double-shelled SA11 virus purified on CsCl gradients (Mason et al., 1980) as follows: a 40 μl amount of anti-SA11 serum diluted 1:100 was mixed with 360 μl virus (260 μg/ml) or 0.01 M-phosphate-buffered saline (PBS), then incubated 1 h at 37 °C and overnight at 4 °C. The immune complexes were removed by centrifugation in a Beckman airfuge at 20 lbf/in² (138 kPa) for 15 min. No peroxidase staining occurred in infected cells incubated with absorbed serum, while mock-absorbed serum retained this activity.

Monospecific antisera were prepared against VP7, the 38 000 mol. wt. (38K) protein, the major glycoprotein in the outer capsid layer; VP6, the 41 000 mol. wt. (41K) protein, the major inner capsid protein; VP2, the 94 000 mol. wt. (94K) protein, the second most abundant inner capsid protein by injecting guinea-pigs with purified peptides eluted from SDS-polyacrylamide gels. The nomenclature of the SA11 peptides used here is that described by Estes et al. (1981). Specificity of the monospecific antisera was confirmed by immunoprecipitation of only the indicated proteins from infected cell lysates (Ericson et al., 1982; H. Hanssen and M. K. Estes, unpublished results). The anti-VP7 serum has been shown to precipitate with equal efficiency either the glycosylated protein or the unglycosylated precursor protein detected only after treatment of infected cells with tunicamycin (Ericson et al., 1982). Preimmune sera from all of the immunized guinea-pigs failed to react with SA11-infected cells in immunoperoxidase procedures and did not precipitate any proteins from SA11-infected cells.

Ultrastructural immunocytochemistry. Confluent monolayers of MA104 cells in 35 mm plastic culture dishes were washed three times in Tris-buffered saline (TBS) and inoculated with 5 to 10 p.f.u./cell SA11. After 1 h adsorption at 37 °C, cells were washed three times with TBS to remove unadsorbed virus and incubated at 37 °C. At various times after adsorption, cells were washed twice with PBS pH 7.4, and fixed for 7 min in 1% 1-ethyl-3-(3-dimethyl-
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RESULTS

Immunocytochemistry using hyperimmune anti-SA11 serum

SA11-infected cell cultures were processed for ultrastructural localization of virus antigens between 18 and 24 h post-infection, a time when virus-induced cytological alterations were widespread. At low magnifications (<×10000) a definite, overall cytoplasmic staining could be seen (Fig. 1a), while rotavirus antigens appeared to be absent from the nuclei of infected cells. The structures most heavily labelled by anti-SA11 serum were viroplasmic inclusions, virus particles and the membranes of virus-associated cisternae. Many of the infected cells had reduced cytoplasmic density, as observed previously in rotavirus-infected cells (McNulty et al., 1976; Pearson & McNulty, 1979), and some cells were probably lysing at the time of fixation. The immunological reagents penetrated lysing cells readily and reacted with a variety of host cell membranes, including the nuclear and outer mitochondrial membranes (Fig. 1b), as well as with virus-induced structures. The more intense staining seen at the periphery of the viroplasms in Fig. 1(b) was commonly observed with the anti-SA11 serum. At higher magnifications, the outlines of developing virus particles could occasionally be seen within viroplasmic inclusions (see Fig. 2a). No peroxidase reaction product was observed in SA11-infected cells incubated with preimmune guinea-pig serum (Fig. 1c), or in mock-infected cells incubated with the hyperimmune anti-SA11 serum (Fig. 1d) or any of the other immune sera.

By scanning a large number of infected cells, we were able to observe that several of the virus-induced structures which occurred relatively rarely (e.g. in 1 to 10% of the cells in a particular experiment) were specifically labelled with anti-SA11 sera. These included bundles of cytoplasmic tubules or filaments probably corresponding to the 15 to 20 nm diam. tubules previously...
Fig. 1. SA11-infected MA104 cells processed for immunocytochemistry. (a) Intact cell labelled with anti-SA11 serum by the PAP technique; (b) lysing cell labelled with anti-SA11 serum by indirect immunoperoxidase (IP); (c) cell labelled with preimmune guinea-pig serum by the IP technique; (d) mock-infected MA104 cell reacted with anti-SA11 serum. Bar markers represent 1 μm. Abbreviations in this and subsequent figures are: Nu, nucleus; er, endoplasmic reticulum; m, mitochondria; vi, viroplasmic inclusion; vp, virus particles.
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Fig. 2. Virus-induced structures in SA11-infected cells labelled with anti-SA11 serum using the PAP procedure. (a) Viroplasmic inclusion and virus particles within the endoplasmic reticulum, arrowheads indicate developing particles; (b) convoluted smooth membrane (sm) within the endoplasmic reticulum; (c) aberrant, elongated virus-like particles in the infected cell cytoplasm; (d) bundle of 15 to 20 nm diam. tubules in the cytoplasm, arrowheads denote individual tubules. Bar markers represent 0.5 μm.

reported in SA11-infected cells (Lecatsas, 1972; Altenburg et al., 1980) (see Fig. 2d), larger tubules (70 nm diam.) with a dense core which resembled elongated virus particles (Fig. 2c) and convoluted smooth membrane material within some of the virus-filled cisternae (Fig. 2b).

Because the fixation procedures used in these experiments did not preserve the plasma membrane of infected cells well enough to evaluate their antigenicity, we attempted to label the outer membranes of unfixed (living) and formaldehyde-fixed cells with the anti-SA11 serum using PAP and with anti-SA11 immunoglobulins bound to colloidal gold (Geoghegan & Ackerman, 1977). No external cell surface antigens could be detected by these methods, although extracellular virus particles were specifically labelled (data not shown), suggesting that SA11 antigens are not inserted into the plasma membrane although virus proteins may be associated with its internal surface.

Immunocytochemistry with monospecific antisera to isolated structural polypeptides

Results obtained using antiserum to VP7, the 38K outer shell glycoprotein (Fig. 3a, b), were different in several respects from those described for the multivalent anti-SA11 serum. The viroplasmic inclusions reacted poorly with anti-VP7, and the general cytoplasmic staining (ribosomes, various membranes) was less intense in relation to the virus particles and endoplasmic reticulum enclosing them, which were heavily labelled (compare Fig. 3a, b with Fig. 1a). The nuclear membrane, which is often associated with virus particles, also reacted with the anti-VP7 serum in many cells (Fig. 3b). Close examination of the virus particles in Fig. 3(b) reveals that they are stained primarily on the external surface, in keeping with the location of glycoprotein VP7 in the outer capsid.

In contrast to the reaction pattern observed with anti-VP7, monospecific antiserum to VP6, the most abundant inner capsid protein, stained viroplasms intensely and uniformly (Fig. 4a). The ribosomes and cytoplasmic membranes appeared similar to those in cells stained with anti-SA11 serum. Virus particles which reacted with the anti-VP6 serum (Fig. 4b, c) appeared
Fig. 3. SA11-infected cells labelled with anti-VP7 (outer capsid glycoprotein) serum by the IP procedure. (a) Viroplasmic inclusions and general cytoplasm are weakly stained in comparison to endoplasmic reticulum and virus particles; (b) virus particles are stained primarily at their periphery, the nuclear membrane is also peroxidase-labelled. Bar markers represent 1 μm.

Fig. 4. SA11-infected cells labelled with anti-VP6 (major inner capsid protein) serum. (a) Viroplasmic inclusions and ribosomes are labelled, but virus particles within the endoplasmic reticulum are not; (b) virus particles within the endoplasmic reticulum of a different, but identically processed cell from (a); (c) inset of virus particles shown in (b) at higher magnification. Bar markers in (a) and (b) represent 1 μm, and in (c) 0·25 μm.
Fig. 5. SA11-infected cell labelled with anti-VP2 (inner capsid protein) serum. Viroplasmic inclusions are heavily labelled. Endoplasmic reticulum and virus particles are labelled but are difficult to distinguish from the cytoplasmic background (ribosomes etc.) which also reacted with anti-VP2. Bar marker represents 1 μm.

smaller than those stained with anti-SA11 (Fig. 2a), which is consistent with the inner capsid location of this protein.

Antiserum to VP2, the 94K inner capsid protein, gave a cytoplasmic staining reaction similar to that of anti-VP6 (Fig. 5). Viroplasmic inclusions also reacted well with this serum. However, virus particles did not stain as intensely with anti-VP2 serum as they did with any of the other antisera (Fig. 5) and were difficult to distinguish.

Technical considerations in localizing virus antigens by ultrastructural immunocytochemistry

Antibody penetration was quite variable in SA11-infected cells that retained their cytoplasmic density, a problem also noted by other groups of workers using similar techniques (Bohn, 1980; Chasey, 1980; Stanislawski et al., 1980). Staining of large viroplasms, especially with the anti-SA11 serum, was usually heavier at the periphery than in the centre, suggesting that penetration was not always complete. However, these experiments do not exclude the possibility of differential distribution of antigens. SA11 antigens were not detected in the nuclei of infected cells, even though nuclear inclusions with tubules similar to those in Fig. 2(b) are occasionally observed in SA11-infected cells (Lecatsas, 1972; Altenburg et al., 1980). However, experiments with cells expressing the simian virus 40 nuclear T antigen have indicated that the nuclear membranes are often not permeabilized by the procedures used here (B. Petrie, unpublished results). A similar situation seems to occur occasionally with the rough endoplasmic reticulum membrane (see Fig. 4a), where virus particles within the cisternae are not stained.

Demonstration that the immunocytochemical reactions were not technical artefacts

Glutaraldehyde, a bifunctional cross-linking reagent, was used in the primary fixation prior to the addition of antiserum in the experiments described above. Therefore, it was possible that rotavirus proteins within the cytosol were artificially and non-specifically bound to cellular membranes and to other structures by this procedure. To check this possibility, a subcellular ‘granule fraction’ consisting of mitochondria, endoplasmic reticulum and various membrane-bound vesicles was isolated from SA11-infected cells, incubated with anti-SA11 serum and subsequently with immunological reagents before fixation and further processing. PAP labelling of the granule fraction prior to glutaraldehyde fixation detected the presence of rotavirus antigens in the mitochondria and intracellular membranes (Fig. 6a). The lack of
staining in granules from mock-infected cells (Fig. 6b) and in infected cell granules incubated with preimmune serum (Fig. 6c) demonstrated immunological specificity. As an additional control, a sample of the granules from mock-infected cells was incubated for 2 h at 0 °C with the post-granule supernatant from infected cells and then washed twice in PBS before incubation with anti-SA11. These granules also appeared negative for rotavirus antigens (Fig. 6d).

DISCUSSION

The present work was undertaken to obtain a more detailed understanding of rotavirus morphogenesis than can be achieved by conventional electron microscopy or by immunocytochemical techniques used with light microscopy. Successful ultrastructural immunocytochemical labelling of rotavirus antigens was accomplished before embedding by using a mild detergent, saponin, to permeabilize the cells after a brief pre-fixation procedure. This procedure did minimal damage to intracellular membranes and left most of the cellular organelles easily recognizable. However, many of the cells which were heavily labelled had reduced cytoplasmic density, a common feature of rotavirus-infected cells, which may have been enhanced by leaching during the permeabilization, antibody incubations and washing steps. Since it was primarily in these lysing cells that virus antigens were detected on mitochondrial and other host cell membranes, the possibility of redistribution of virus proteins must be considered. The finding of SA11 antigens associated with partially purified and washed mitochondria from infected cells indicates that their presence was not caused by reagents used during pre-fixation or saponin treatment. It therefore seems likely that the presence of rotavirus antigens in or on cellular structures which are not directly involved in virus replication results from association with soluble proteins or proteins released from the breakdown of viroplasms and virus-containing cisternae during lysis of the host cell.

The present study confirmed that several cytoplasmic structures specifically induced by SA11 infection of cultured cells are composed of virus structural antigens. As expected, the dense granular inclusions, believed to be the site of virion assembly, reacted strongly with anti-SA11 serum. In areas where the penetration of the immunoperoxidase reagents was extensive, outlines of partially assembled virus particles were visible within these viroplasmic inclusions. The 15 to 20 nm diam. cytoplasmic tubules were also noted to contain virus antigens, suggesting that they
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Table 1. Use of monospecific antisera to detect differential distribution of rotavirus antigens within infected cells

<table>
<thead>
<tr>
<th>Structures</th>
<th>Anti-SA11</th>
<th>Anti-VP2</th>
<th>Anti-VP6</th>
<th>Anti-VP7</th>
<th>Interpretation</th>
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<tr>
<td>Viroplasmic inclusions</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>±</td>
<td>Assembly of single-shelled particles</td>
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<tr>
<td>Virus particles</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>Staining reflects location of proteins in particles</td>
</tr>
<tr>
<td>Endoplasmic reticulum membrane</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Synthesis of VP7, assembly of outer shell</td>
</tr>
<tr>
<td>Other virus-induced structures</td>
<td>+</td>
<td>ND*</td>
<td>ND</td>
<td>ND</td>
<td>Composed of structural protein(s)</td>
</tr>
</tbody>
</table>

*ND, Not detected.

may result from unbalanced synthesis of one or more SA11 proteins. We were unable to determine whether the 38K glycoprotein, the 41K protein or the 94K protein were components of these tubules, as they were not seen in any of the cells reacted with antibody to these polypeptides. Although rare in rotavirus-infected cells, similar appearing tubules or fibres are commonly seen in cell cultures infected with members of the related orbivirus group. However, in contrast to our data with SA11, they are thought to be composed of non-structural virus-specified proteins (Oshiro & Emmons, 1968).

A second type of tubule similar to one reported in SA11 infection of primary rhesus monkey kidney cells (Altenburg et al., 1980) and to those seen in cells infected with EDIM virus (Banfield et al., 1968) was observed in close proximity to the virus particles in some of the SA11-infected cells. As these tubules were the same diameter as the particles, contained an electron dense core structure and were heavily coated with the immune reaction products, they probably represent aberrant virus assembly. Accumulations of smooth membrane-like material within virus-containing cisternae also reacted with anti-SA11 serum, suggesting that these membranes may be similar in composition to virus envelopes.

The use of monospecific antisera to three major polypeptides of the capsid allowed us to detect a differential distribution of rotavirus antigens within infected cells. A summary of these findings is presented in Table 1. The two inner capsid proteins, VP6 and VP2, were concentrated in the viroplasmic inclusions, where assembly of rotavirus particles is believed to occur. However, these inclusions were only weakly stained with antiserum to VP7, the outer capsid glycoprotein, suggesting that this protein is present in smaller quantities than the other proteins. It is also of interest that antiserum to VP2, the 94K protein, stained virus particles rather weakly (Fig. 5). Recent evidence from work with the calf rotavirus (Bican et al., 1982) has shown that VP2 protein is associated with an inner ‘core’ structure and therefore might be less accessible to antibody than either VP6 on the inner shell or VP7 on the outer shell.

The data from these experiments suggest that rotavirus proteins are synthesized both on the free and membrane-bound ribosomes. The antisera to VP6 and VP2, as well as the multivalent anti-SA11 serum, reacted with ribosomes distributed throughout the cytoplasm of infected cells. However, the glycoprotein was associated primarily with ribosomes of the rough endoplasmic reticulum. These data are consistent with other experiments (McCrae & Faulkner-Valle, 1981; Ericson et al., 1982), suggesting that glycosylation of VP7 occurs co-translationally in the lumen of the endoplasmic reticulum. This pattern of glycoprotein synthesis is known to occur in a wide variety of enveloped RNA viruses (Klenk & Rott, 1980) in which the glycoproteins are major constituents of the virus envelope. Rotaviruses are unique in that the infectious virions are not enveloped, although they initially acquire an envelope derived from the endoplasmic reticulum (Petrie et al., 1981). It seems likely that the 38K glycoprotein is added to the outer capsid layer during the enveloped stage of virus assembly.

Our results demonstrate the usefulness of combining electron microscopic cytochemical techniques with highly specific immunological probes to study virus replication and assembly at
the molecular level. Further experiments with the antisera described here, as well as with antisera being prepared to other SA11 structural and non-structural proteins, should allow us to determine the polypeptide composition of the many rotavirus-induced structures and help identify the function of each protein in morphogenesis.

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