Isolation and Partial Characterization of Two Forms of Cytoplasmic Nucleocapsids from Measles Virus-infected Cells

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SUMMARY

Two species of measles virus nucleocapsids with distinct buoyant densities were isolated from infected AVa cell homogenates by isopycnic CsCl gradient centrifugation. The more buoyant or ‘light’ form of the nucleocapsid had a density of 1.26 to 1.28 g/ml, whereas the less buoyant or ‘heavy’ nucleocapsid species had a density of 1.30 g/ml. Analysis of the two nucleocapsid species by SDS–polyacrylamide gel electrophoresis showed that both forms possessed two phosphorylated polypeptides with mol. wt. of 69,000 and 60,000. The heavy form of nucleocapsid consisted solely of these two polypeptide species, while the light form of nucleocapsid had two additional associated polypeptides (VP4, mol. wt. 52,000, and 45K, mol. wt. 45,000). Ultrastructural and immunofluorescent studies suggest that the two isolated capsid forms represent the two morphologically distinct capsid species observed in vivo. This paper discusses the possible relationship between the two capsid forms and assembly of the virus.

INTRODUCTION

Measles virus particles contain six major structural polypeptides: VP1, haemagglutinin (HA); VP2, nucleocapsid-associated protein (P); VP3, nucleocapsid protein (NP); VP4; VP5, fusion protein or haemolysin (F1 or HL); and VP6, membrane protein (M) (Bussell et al. 1974; Mountcastle & Choppin, 1977; Hardwick & Bussell, 1978; Tyrrell & Norrby, 1978; Wechsler & Fields, 1978; Robbins & Bussell, 1979). In addition to these proteins, the virus contains at least three minor polypeptide components (L, a high mol. wt. capsid-associated protein; cellular actin; and F2, a small mol. wt., highly glycosylated fragment of the virus fusion protein; Hardwick & Bussell, 1978; Tyrrell & Norrby, 1978; Vainionpää et al. 1978; Robbins et al. 1980). Although a number of investigations have been conducted on the structural proteins of the virus, few studies have examined the capsid proteins in detail. Mountcastle & Choppin (1977) and Robbins & Bussell (1979) have previously shown that the nucleocapsids of measles virus possess two major structural proteins (VP2 [P] and VP3 [NP]). More recently, at least two minor capsid-associated polypeptides (L and VP4) have been described (Stalleup et al. 1979; Robbins et al. 1980). These studies indicate that the capsid proteins of measles virus are similar to those reported for other paramyxoviruses (Marx et al. 1974; Colonno & Stone, 1976; Buetti & Choppin, 1977).

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Ultrastructural studies conducted by Matsumoto (1966), Oyanagi and co-workers (1971) and Dubois-Dalcq and associates (1973, 1974a, b) have clearly demonstrated that cells infected with measles or subacute sclerosing panencephalitis (SSPE) virus possess two forms of virus capsid, 'fuzzy or granular' and 'smooth'. This is in contrast to the single form of capsid found in the cytoplasmas of cells infected with other paramyxoviruses (Compans et al. 1966; Duc-Nguyen & Rosenblum, 1967; Howe et al. 1967; McLean & Doane, 1971). The relationship between the capsid proteins described in the biochemical studies and the two forms of virus nucleocapsid observed in the ultrastructural studies has not been determined.

This paper describes the purification of two cytoplasmic forms of measles virus nucleocapsid and the characterization of their associated polypeptides. Biochemical and ultrastructural evidence obtained from these studies suggest that the two species of capsids isolated represented the two morphologically distinct capsid forms observed in vivo.

METHODS

Cells and media. Human amnion cells (AV₃) used in this study were maintained as previously described (Robbins & Bussell, 1979) with the following modifications: (1) cell dispersant solutions employed in cell passage consisted solely of 0.02%, w/v, EDTA in calcium and magnesium-free phosphate-buffered saline (CMF-PBS), and (2) Eagle's (modified) minimum essential medium (Flow Laboratories, Rockville, Md.) with 2 mM-L-glutamine, and 10%, v/v, calf serum was used for cell passage.

Virus. The passage history and characteristics of the Edmonston measles virus strain used in these studies have been previously described (Bussell & Karzon, 1965; Robbins & Bussell, 1979).

Infection and labelling procedures. The methods employed for infecting and radioisotopic labelling of cell cultures have been described elsewhere (Robbins & Bussell, 1979). Cell cultures were infected at 0.1 p.f.u./cell in all studies described here.

Nucleocapsid isolation from infected cells. Twenty-four hours after labelling, infected cell monolayers were washed twice with warm CMF-PBS and incubated with a warm 0.02% solution of EDTA in CMF-PBS at 37ºC for 30 min. Cells were removed from the surfaces of roller bottles by gently swirling the chelation solution over the monolayers. Cells were harvested by low-speed centrifugation (2000 rev/min in an International clinical centrifuge) and the cell pellet was resuspended in 8 to 10 ml of cold 20 mM-Na₂CO₃ and 2%, v/v, Brij-35 detergent (Sigma Biochemicals, St. Louis, Mo.), pH 11. The cells were allowed to swell in the hypotonic solution for 15 min and the suspension was then homogenized with 10 to 15 strokes of a tight fitting Dounce homogenizer. The homogenate was centrifugally clarified at 4ºC in a refrigerated RC2–B Sorvall centrifuge at 7500 rev/min (6800 g) for 5 min using a SS–34 fixed angle rotor. The clarified cytoplasmic extract was processed according to the methods of McSharry et al. (1975) and Compans & Choppin (1967) with certain modifications. The procedure employed involved the isopycnic banding of nucleocapsid materials on a series of three gradients. The first gradient consisted of 5%, w/v, sucrose and 25 to 40%, w/w, CsCl in 20 mM-Na₂CO₃, pH 10, and was centrifuged in a SW–27 Beckman rotor at 24000 rev/min for 3 h at 4ºC. Visible bands of material in the 30%, w/w, CsCl region were harvested, diluted in 20 mM-Na₂CO₃ and re-banded on gradients of 25 to 40%, CsCl in carbonate buffer as before. Bands from the second gradients were isopycnically banded a third time on identical gradients. The third gradients were fractionated and samples were counted in a 3% solution of Protosol (New England Nuclear,
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Boston, Mass.) in Omnifluor-toluene scintillant (New England Nuclear). Aliquots of each fraction (100 μl) were also weighed to determine densities. Fractions containing the labelled materials were dialysed overnight against distilled water at 4 °C. Dialysed material was pelleted in a SW-41 Beckman rotor at 35,000 rev/min for 60 min at 4 °C and the pellets were resuspended in a small volume of 20 mM-Na2CO3. A sample of the resuspended radio-labelled materials was counted in a Beta-scintillation counter and the remaining suspension was frozen at −70 °C until used. Protein present in each sample was determined by the method of Lowry et al. (1951).

Polypeptide analysis by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Polypeptides associated with labelled and unlabelled nucleocapsid materials were identified by SDS-PAGE through 5% polyacrylamide disc gels in a phosphate-buffer system as described elsewhere (Maizel, 1971). All samples were electrophoresed under reducing conditions. Labelled gels were processed as described previously (Robbins & Bussell, 1979). Scanning gels were stained with 0.5% Coomassie brilliant blue R-250 (Sigma Biochemicals, St. Louis, Mo.), destained in a solution of 14% acetic acid and 50% methanol and scanned at 620 nm on a densitometer. The mol. wt. of the capsid-associated proteins were determined by electrophoresing marker proteins with known mol. wt. (Sigma Biochemicals) on companion gels for comparison of their relative migrations.

Electron microscopy. Measles virus-infected cells were harvested and fixed in a 3% glutaraldehyde, 200 mM-cacodylate solution, 48 to 72 h p.i. The cells were postfixed in 1% OsO4, embedded in agar and stained with 2% uranyl acetate. The samples were then dehydrated in a series of alcohol and propylene oxide, embedded in Epon resin and thin-sectioned. After staining the thin sections with 2% uranyl acetate and 1% lead citrate, they were scanned with either an EMU-3 RCA or Hitachi HU-I2 model electron microscope. Samples of purified nucleocapsid were treated similarly except they were not pre-embedded in agar.

RESULTS

Purification of cytoplasmic nucleocapsid species

When clarified cytoplasmic extracts of infected AV3 cells were processed on a CsCl-sucrose gradient, two distinct bands were consistently seen in the 30%, w/w, CsCl region (Fig. 1 a). Following the final gradient purification step, the light band appeared as a well-resolved band of fine consistency and the heavy band appeared as an aggregate of amorphous clumps (Fig. 1 b and c). Subsequent gradient fractionation and density characterization showed that the light band had a density of 1.26 to 1.28 g/ml, while the heavy band had a density of 1.30 g/ml (Fig. 2). Extensive isopycnic centrifugation of the nucleocapsid materials did not alter the buoyant density of either the light or heavy bands, suggesting that each represented separate species of intracellular nucleocapsids. When dialysed samples of the banded materials were measured for total protein by the method of Lowry et al. (1951), the amount of protein in the lighter band was consistently greater than the amount found in the heavy band. This suggested the possibility that the lighter band represented the more prevalent form of capsid observed in the ultrastructural studies described below. We have not, as yet, undertaken analyses of the RNA component of either the heavy or light capsid species. However, results of SDS-PAGE studies (also described below) suggest that the density differences between the two capsid species are, at least in part, the result of differences in protein composition.
Fig. 1. Density gradients of cytoplasmic extracts and nucleocapsid materials from acutely infected AV3 cells: (a) CsCl-sucrose gradient of clarified cytoplasmic extracts (Gradient 1); (b) isopycnic CsCl gradient of purified light nucleocapsid materials; (c) isopycnic CsCl gradient of heavy nucleocapsid materials. Abbreviations CV, L, and H refer to cell-associated virus, light nucleocapsid and heavy nucleocapsid respectively.

Fig. 2. Density profiles of cytoplasmic nucleocapsid materials on isopycnic CsCl gradients. (a) Light nucleocapsid materials ($\rho = 1.26$ to $1.28 \text{ g/ml}$); (b) heavy nucleocapsid materials ($\rho = 1.30 \text{ g/ml}$). Sedimentation is from right to left. $\triangle$, Density, g/ml.
Fig. 3. Electron micrographs of measles virus nucleocapsid species. (a) Cytoplasmic fuzzy nucleocapsids and (b) intranuclear smooth nucleocapsids in infected AV₃ cells. (c) Light nucleocapsids and (d) heavy nucleocapsids purified from infected AV₃ cells. Arrows in micrographs (a) and (c) delineate representative capsid structures.

**Ultrastructural studies**

Previous studies conducted by a number of other workers (Matsumoto, 1966; Oyanagi et al., 1971; Dubois-Dalcq & Barbosa, 1973; Dubois-Dalcq et al., 1974a, b) have shown that cells infected with measles and SSPE viruses possess two forms of virus nucleocapsid: (1) a fuzzy or granular form found exclusively in the cytoplasm of infected cells with a diam. of 25 to 30 nm, and (2) a smooth form which appears to be a simple helical structure approx. 18 nm in diam. and is found in both the cytoplasm and the nucleus. These studies also demonstrated that the formation of mature measles virus particles involves the association of the wider form of nucleocapsid with regions of the host cell membrane and the subsequent release of the enveloped complexes by a budding process.

To test whether these two forms of capsid were also present in infected AV₃ cells, infected
cells were fixed and embedded in Epon resin and thin sections were examined under the electron microscope. Such cells possessed abundant cytoplasmic tubular structures which the uninfected control cells did not appear to have (data not shown). Upon closer examination, two forms of virus capsid were apparent in the infected cells. The prevalent form of capsid was found exclusively in the cytoplasm and appeared to be composed of a virus capsid covered with a thick coat of granular material of 'fuzz' (Fig. 3a). This form appeared to be found most frequently in the peripheral regions of the cell and was structurally indistinguishable from the capsids leaving the cell surface in evaginating blebs. The other form of capsid (smooth) appeared in both the cytoplasm and the nucleus but was seen much less frequently than the fuzzy form. These structures appeared as well-resolved herringbone-like tubules which were occasionally found in paracrystalline-like aggregates (Fig. 3b). When smooth nucleocapsids were observed in the cytoplasm, they were found almost exclusively in the perinuclear region (data not shown).

To determine whether the two bands of capsid isolated from infected cells were structurally similar to the two cytoplasmic capsid species seen in infected cells, thin sections of the purified materials were examined by electron microscopy. As is evident in Fig. 3, the purified capsid species were remarkably similar to the two forms of capsids observed in vivo: the light form possessing a fuzzy coat of material (Fig. 3c) and the heavy form lacking it (Fig. 3d). The similarity of the capsid diameters in vivo and in purified capsid preparations supports the hypothesis that the light form of capsid represents the fuzzy in vivo form and the heavy capsid species represents the smooth in vivo form.
Polypeptide composition of cytoplasmic nucleocapsids

SDS-polyacrylamide gels of the purified capsid materials were run to determine their respective polypeptide compositions. Analysis of the polypeptides of labelled intracellular nucleocapsids by SDS-PAGE showed distinct differences between the two species (Fig. 4). While both forms of nucleocapsid contained two major polypeptide components (VP2 and VP3) which appeared to be present in similar ratios, the light form contained additional polypeptides not contained by the heavy form. The presence of two additional proteins (VP4; mol. wt. 52,000 and 45K; mol. wt. 45,000) on the light form of nucleocapsid was evident in labelled profiles but was more dramatically demonstrated in stained gels (Fig. 5). The discrepancy between the relative amounts of VP4 and 45K on the light capsid species in the labelled polypeptide profiles (Fig. 4a) and stained profiles (Fig. 5a) suggests that these two polypeptide species were of cellular instead of virus origin.

Interestingly, we did not observe the L polypeptide in any of our capsid preparations. While this is in agreement with certain previous studies conducted on measles virus nucleocapsids (Mountcastle & Choppin, 1977; Robbins & Bussell, 1979), it is in conflict with a more recent report by Stalleup et al. (1979) in which the L polypeptide was consistently found in association with isolated measles virus cytoplasmic nucleocapsids. Although we are uncertain as to the exact reason(s) for this discrepancy, we believe the absence of the L polypeptide in our capsid preparations may possibly be due to the repeated exposure of the capsids to the high salt (CsCl) concentrations of the density gradients.

In an earlier communication, the two major capsid proteins of measles virus, VP2 and VP3, were identified as phosphoproteins (Robbins & Bussell, 1979). Based on the observation that VP2 and VP3 appeared to be phosphorylated in both forms of cytoplasmic nucleocapsid (see Fig. 4), experiments were performed to determine (1) whether there were differences in the specificity of phosphorylation (i.e., in vivo phosphate-acceptor amino
acids) between the phosphoproteins of the two forms of capsid, and (2) whether any differences in the phosphorylation patterns could account for the association of the extra polypeptides with the light form of nucleocapsid. Identification of the phosphoamino acids in VP2 and VP3 from light and heavy nucleocapsids was accomplished by methods previously described (Robbins & Bussell, 1979).

Scintillation profiles of the phosphorylated amino acid species in the two major polypeptides from each form of nucleocapsid are shown in Fig. 6. While serine was the primary phosphorylated amino acid in all the polypeptides, significant, but lesser, amounts of phosphothreonine were also present in VP3. Since the relative amounts of phosphoserine and phosphothreonine are similar for VP2 and VP3 from either form of nucleocapsid, it appeared that differential phosphorylation played no role in the acquisition of the extra polypeptides by the light form of nucleocapsid.

**DISCUSSION**

Evidence acquired in this study suggests that the two forms of nucleocapsids isolated represent the two capsid species observed in ultrastructural studies. Data which support this observation include: (1) differences in polypeptide composition between the two forms of capsid, with one form of capsid possessing proteins which the other form does not; (2) yields of the two nucleocapsid species which, when harvested, reflect the populations of capsids in infected cells observed under the electron microscope; and (3) distinct ultrastructural similarities between the in vivo capsid forms and the forms purified from infected cells. Our studies also indicate that the fuzz on the fuzzy capsids may be composed of the two polypeptide species found on the light form of capsid (VP4 and 45K). Based on the results of our gel studies, it seems likely that these two proteins are of a cellular, rather than of virus, origin.

The identities of the proteins we have designated VP4 and 45K remain obscure. Based on
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Previous ultrastructural and biochemical studies, it appears possible that these proteins may be involved in the assembly of measles virus particles since they are found in 'purified' virus preparations (Bussell et al. 1974; Tyrrell & Norrby, 1978; Vainionpää et al. 1978). Whether the association of these polypeptides with cytoplasmic capsids is of structural or mechanical significance (as in the intracytoplasmic transport of perinuclear capsids to peripheral regions of the cell) remains to be determined.

The light form of capsid does not appear to represent an artifact of our purification process for the following reasons: (1) mixtures of heavy capsids and uninfected cell extracts did not result in the formation of a light capsid species when such suspensions were centrifuged on isopycnic CsCl gradients, and (2) only heavy capsids (ρ = 1.30 g/ml) have been obtained from cytoplasmic extracts of persistently infected AVa cells (a cell line in which only smooth capsids are apparent in ultrastructural studies; S. J. Robbins & R. H. Bussell, unpublished observations). Although we believe that the light (fuzzy) capsids are, in fact, only modified heavy (smooth) capsids, we are not certain by what mechanism this assembly or association takes place. Our data suggest that phosphorylation of the two major capsid structural proteins (VP2 [P] and VP3 [NP]) is not involved.

It is perhaps significant that throughout these studies we have not detected the virus membrane protein (VP6 or M; 37,000 mol. wt.) in any of our capsid preparations. While it is possible that our purification processes stripped this polypeptide from one (or both) of the capsid species we have isolated, it seems more likely that the M protein was not associated with the capsids until the very final stages of the virus assembly process (membrane association of capsids and evagination of virus particles). Evidence for this type of rapid association of membrane protein with maturing virions has been described previously for another paramyxovirus, Sendai virus (Portner & Kingsbury, 1976). However, until similar definitive molecular studies are conducted on the measles virus assembly process, our knowledge of this stage of the virus replicative cycle will remain incomplete.

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