Isolation and Characterization of a Membrane-Bound Population of Group B Coxsackieviruses

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Received 15 September 1982/Accepted 10 November 1982

HeLa cells infected with several group B coxsackieviruses contain a previously undetected, virus-specific ribonucleoprotein particle which we designated membrane-bound virion (MBV). MBVs of B5 virus have a pronounced polygonal appearance and are slightly smaller than virions. The particles sediment more slowly (at about 107S) and have a lower buoyant density (about 1.30). They contain 35S virion RNA; only three, and not four, capsid proteins; and at least seven additional proteins with apparent molecular weights of 21,000 to 92,000. Three of the latter proteins appear to be of host origin; the rest may be precursors of virion capsid proteins. The RNA is resistant to digestion by RNase, and EDTA treatment disrupts the particle. MBVs are infectious, although significantly less so than virions. Cells infected with MBVs produce both types of progeny, virions and MBVs. In coinfect ed cultures, the yield of progeny is lower than in cells infected with virions alone, suggesting interference by MBVs. Synthesis of both types can be detected within 3.5 h after infection, and synthesis continues for 24 h.

Group B coxsackieviruses (B1-6), along with several small RNA viruses such as group A coxsackie, poliovirus, echovirus, and a number of enteric viruses from livestock and other animals, belong to the genus Enterovirus of the family Picornaviridae (27, 30). A number of capsid-related structures which are thought to be intermediates of virion morphogenesis have been isolated from picornavirus-infected cell extracts (30). In the case of poliovirus, the most-studied member, several forms of the capsids are found: 156S to 160S virions, 75S to 80S empty capsids without RNA (28), and 14S particles capable of self-assembly into 74S particles in cell-free extracts (26).

Another intermediate in poliovirus virion synthesis, the proviron, sediments at about 125S (9) or, in some preparations, at about 150S (12) and contains virion capsid proteins VPO, VP1, and VP3 as well as 35S viral RNA. It is sensitive to EDTA and high salt concentrations. Proviron formation in cell-free extracts has been detected (10). Capsid-related empty particles, but not provirions, have also been identified in other picornaviruses, such as echovirus (13), foot-and-mouth disease virus (29), and human rhinovirus (18).

In the case of group B coxsackievirus, practically no direct information is available on naturally occurring, capsid-related particles. B3 virions sediment at about 150S; treatment of these with 1 M urea generates 75S to 80S procapsids (lacking capsid protein VP4) and 20S and 5S particles (25). Upon further urea treatment, the procapsids generate 40S particles (containing mostly VP0) and 20S particles (containing only VP1 and VP3). These structures, however, have not been isolated from the infected cells.

During our investigations of group B viruses, we isolated, in addition to mature virions, a previously unrecognized coxsackievirus-specific ribonucleoprotein particle from infected cells. The particle, which we call the membrane-bound virion (MBV), since it contains host cell cytoplasmic membrane proteins, is present in B2-, B4-, B5-, and B6-infected HeLa cells. It contains all but one of the virion capsid proteins and viral RNA. It also contains several additional proteins, some of which appear to be of host origin and others which are capsid protein precursors. MBVs sediment slower, have a lower buoyant density, and are slightly smaller than virions. They are infectious, although significantly less so than virions, and appear to inhibit infection produced by the virions. In most of the experiments we report, B5 virus was used.

MATERIALS AND METHODS

Viruses and cells. Growth of HeLa S-3 and Vero cells and growth and plaque assay of coxsackieviruses B2 (Ohio Red), B4 (Van Barscholten), B5 (Faulkner), and B6 (Schmitt) have been described previously (4).

Labeling of infected cells and virus purification. HeLa cells were concentrated to about 10⁷ cells per ml, infected with 0.2 to 5 PFU of virus per cell, and incubated at 37°C. To label virus after adsorption, the cells were pelleted by centrifugation at 2,000 × g for 10
min and resuspended in HEPES (4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid)-buffered Eagle minimum essential medium containing 1/10 of the normal amount of methionine and 5% dialyzed fetal bovine serum. After about 1 h of incubation, [35S]methionine (10 μCi/ml, 800 to 1,000 Ci/mmol) and [3H]uridine (2 to 5 μCi/ml, 40 to 50 Ci/mmol) were added, and the incubation was continued. For labeling with 32P, cells were grown in phosphate-free Joklik-modified F-13 medium (GIBCO Laboratories, Grand Island, N.Y.) overnight and labeled with 8 to 10 μCi of carrier-free [32P]Pi per ml.

At 20 h postinfection (p.i.), or sooner where stated, the infected cells were harvested, and the virus was purified by modifications of a published procedure (4). The cell suspension was centrifuged as described above, and the supernatant was saved. The pellet was suspended in a small volume (5 to 10 ml) of phosphate-buffered saline (PBS) containing 0.8% Nonidet P-40, disrupted by 12 to 15 strokes in a Dounce homogenizer with a type B pestle, and centrifuged. This supernatant was combined with the initial one. The extraction procedure was repeated at least once more.

To pellet the virus, the combined supernatants were centrifuged at 150,000 × g for 2 h at 5°C in a Spinco 60 Ti rotor. The pellet was suspended by homogenization in 4 to 6 ml of PBS, incubated at 4°C for 16 h, homogenized while Nonidet P-40 was added to 0.5%, and centrifuged at 5,000 × g to remove insoluble debris. The clarified virus suspension was layered over an 8-ml linear gradient of CsCl in PBS (1.2 to 1.4 g/ml) and centrifuged at 38,000 rpm for 3 h at 6°C in a Spinco SW41 Ti rotor.

Fractions (0.5 ml) collected from the bottom were assayed for trichloroacetic acid-precipitated radioactive activity, and the fractions containing peak radioactivity were pooled. The pooled material was dialyzed against PBS for 16 h at 4°C and recentrifuged in the CsCl gradient as described above. Fractions were collected as described above, and their radioactivity, the ratio of their absorbance at 260 and 280 nm (A260/280), and their refractive index were measured. The one or two fractions containing the virus, as determined by radioactivity, A260/280, buoyant density (ρ), and infectivity, were dialyzed as before and stored in the vapor phase of a liquid nitrogen freezer. In some cases, the virus was purified further by a third centrifugation in CsCl gradient. Extraction of infected cells with Triton or sodium dodecyl sulfate yielded less virus than extraction with Nonidet P-40.

Sucrose density gradient analysis of virus. The sedimentation profile of CsCl gradient-purified virions was analyzed by modifications of a published procedure (1). Virus diluted with 20 mM Tris-hydrochloride (pH 7.6)-10 mM NaCl-1.5 mM MgCl2-pH m dithiothreitol was centrifuged at 34,000 rpm for 3 h at 20°C. The sucrose solution contained Tris-hydrochloride and dithiothreitol as described above, 100 mM NaCl, and 3 mM MgCl2. Polyribosomones (20S) and monoribosomones (80S) from Ehrlich ascites tumor cells were centrifuged as markers in a parallel gradient. Samples of gradient fractions were counted for acid-precipitable radioactivity or used for A260 measurements.

Extraction and characterization of viral RNA. Viral RNA extraction, (5), analysis by velocity sedimentation in 15 to 30% sucrose gradients (5), and analysis in agarose gels containing 6 M urea (21) were performed as previously described.

For T1 oligonucleotide fingerprinting, the RNA was dissolved in a small volume (30 to 50 μl) of 10 mM Tris-hydrochloride (pH 7.5)–2 mM EDTA, boiled for 30 s, and rapidly cooled for 30 s. The RNA was digested with RNase T1 (0.9 U/μg of RNA; Calbiochem, La Jolla, Calif.) for 40 min at 37°C, dried, dissolved in 30 μl of 4.5 M urea–25% glycerol and a trace amount of tracking dye mixture (bromophenol blue, xylene cyanol, and acid fuchsin), and electrophoresed.

The gel electrophoresis procedure has been described in detail previously (7, 20). We modified it to suit our purpose. Electrophoresis was carried out in the first dimension in a gel apparatus (38 by 8 cm) and in the second dimension in another apparatus (34 by 43 cm), both of which were built in our workshop. The first dimension was performed on 14% polyacrylamide gel slabs containing 6 M urea at pH 3.3 at 400 V for 20 h at room temperature. The second dimension was performed on 22% gel slabs with 6 M urea at pH 8.2 and 500 to 600 V, also for 20 h at room temperature. After electrophoresis, the gel was wrapped in Saran Wrap and exposed to Kodak X-Omat AR5 film with an intensifying screen at 70°C.

**Gel electrophoresis of proteins.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (22) in a discontinuous Tris-glycine (pH 8.3) buffer system for virus protein analysis has been described. Electrophoresis was carried out in 12.5% slab gels (1.5 mm thick) at 50 to 75 V until the tracking dye had migrated to a fixed distance. The gels were stained, destained, dried, and autoradiographed (19). The following five methyl-14C-labeled proteins (Amersham Corp., Arlington Heights, Ill.) were electrophoresed on the same gels as molecular weight markers (molecular weights are given in parentheses): phosphorylase b (92,500), bovine serum albumin (69,000), ovalbumin (46,000), carbonic anhydrase (30,000), and lysozyme (14,300).

**Electron microscopy.** Samples of CsCl-purified virus were placed on carbon- and Formvar-coated copper grids, negatively stained with aqueous solutions of 0.5% uranyl acetate, 2% ammonium molybdate, or 2% sodium phosphotungstate (pH 7.0, adjusted with 1 N NaOH), and photographed under an electron microscope. The magnification was determined with a replica grating (6,002×; Ernest E. Fullam, Inc., Schenectady, N.Y.). In micrographs taken at regular intervals during the course of electron microscopy, the variation in magnification was <0.05%.

To establish the size distribution of the virus particles, at least 100 particles from each preparation were measured. Since the particles varied from spherical to polygonal under the influence of neighboring particles, only the longest diameter of each was considered. All measurements were done on photographic enlargements of the negatives; occasional direct measurements from the negatives were used to verify the accuracy of the enlargements.

**RESULTS**

Identification and characterization of MBVs. During virus purification from detergent-extractable cell extracts in CsCl gradients, we consistent-
ly observed two populations of each virus in cells infected with B2, B4, B5, or B6. One population banded at about \( \rho = 1.34 \), and the other banded at about \( \rho = 1.30 \). The former represents the standard virion reported in previous studies (1, 4, 15, 25). The latter, which had not been detected previously, we designated MBV.

Figure 1 shows a composite of several experiments of virus purification from infected or mock-infected cells labeled with two isotopes. \([^{35}S]\)methionine- and \([^{3}H]\)uridine-labeled B5 virus separated as two peaks, I and II, in the first gradient (Fig. 1A). After centrifugation in the second gradient, peak I banded at about \( \rho = 1.34 \) (Fig. 1B), and peak II banded at about \( \rho = 1.30 \) (Fig. 1C). \([^{35}S]\)methionine-labeled B6 also exhibited peaks I and II in the first (Fig. 1D) and second (data not shown) gradients. Peak I banded at about \( \rho = 1.34 \) (Fig. 1E) and peak II banded at about \( \rho = 1.30 \) (Fig. 1F) in the third gradient. In mock-infected cells, very little methionine radioactivity was detected in these regions of the gradients (Fig. 1G through I). The same two populations were observed in B2- or B4-infected cells labeled with methionine and uridine (data not shown). When CsCl gradient-purified B5 virion populations were sedimented through sucrose gradients (Fig. 2), virions (peak I) sedimented at about 150S, and MBVs (peak II) sedimented at about 107S.

MBVs were considerably less infective than virions in the B viruses studied. At about 24 h.p.i. in B5-infected cultures, the infectivity of the
MBVs ranged from $7.6 \times 10^7$ to $2.2 \times 10^9$ total PFU, as compared with $13.5 \times 10^8$ to $2.3 \times 10^{11}$ total PFU for the virions. Thus, infectivity was 18 to 100 times lower in the MBV population. The $A_{260:280}$ was about 1.22 for MBVs, as compared with 1.64 for virions, suggesting that MBVs have relatively more protein than RNA.

To liberate MBVs, it was necessary to extract the infected cells several times with PBS containing 0.5 to 0.8% Nonidet P-40 in a Dounce homogenizer. Simple freezing and thawing of the infected cells several times, the usual procedure for virus extraction, liberated essentially no MBVs. Extraction of freshly harvested cells liberated significantly more virus than extraction of frozen cells.

**Components of MBV.** To analyze the proteins, $^{35}$S methionine-labeled virions of B5 and B6 viruses and mock-infected cell proteins were electrophoresed in polyacrylamide gels (Fig. 3). B5 and B6 MBVs contained more proteins than did the virions. Virions of B5 and B6 contained all four capsid proteins, VP1 to VP4. One of these capsid proteins (to be identified later) was missing in B5 and B6 MBVs. In addition, at least seven additional proteins of apparently 21,000 to 92,000 daltons were also detected in the two MBV populations.

The additional proteins were always present in MBV populations, even after three successive centrifugations in CsCl gradients. They were always absent in the virion populations, except for a trace amount of a 43,000-dalton protein in some preparations of B5. The nature of the additional proteins is not yet known. However, at least three of these proteins (92,000, 67,000, and 56,000 daltons) appeared, from their electrophoretic mobility, to be of host origin. The rest may be precursors of virion capsid proteins.

The RNA of B5 MBV was analyzed by velocity sedimentation in sucrose gradients, migration in 1.5% agarose gels containing 6 M urea, and oligonucleotide fingerprinting after digestion with RNase T1. Virion RNA of B5 virus is a 35S molecule (5). In this study, $^{32}$P-labeled B5 RNA (Fig. 4A) and most of the MBV RNA sedimented at the same position (Fig. 4B). Both RNAs migrated the same distance after electrophoresis in the denaturing gel (Fig. 4B, inset).

The T1 oligonucleotide fingerprint of MBV RNA was distinguishable from that of virion RNA by two oligonucleotides. One in the virion RNA (Fig. 5A, open arrow) could not be detected in MBV RNA. Another, which was always detected in the MBV population (Fig. 5B, solid arrow), could not be detected in some preparations of virions. (Fig. 5A, inset, shows a virion preparation in which this oligonucleotide was detected.) The reason for this discrepancy between virion preparations is unknown.

**Properties of MBV.** To obtain information on the structure of MBV, its stability to RNase treatment was studied. Gradient-purified virions and MBVs were treated with pancreatic RNase (20 µg/ml) for 40 min at 30°C and then plaque assayed. The treatment did not reduce the titer of either population (data not shown), showing that the MBV was stable to the enzyme and indicating that its RNA was protected by the protein.

When purified virions were treated with 20 mM EDTA and sedimented in sucrose gradients, the treatment had no effect, and there was no change in their sedimentation profile (data not shown). Treatment of MBVs with EDTA, however, changed their sedimentation profile, separating the viral RNA from the proteins (Fig. 6). This change suggests disruption of the virions.

To compare the morphology of the virion and
MBV populations, electron microscopic observations were made with B2, B5, and B6 viruses stained with various negative stains as described in Materials and Methods. The results with B5 virus stained with uranyl acetate are reported here.

Most of the virion particles measured between 32 and 33 nm (Fig. 7). MBVs had a broader size distribution and an average diameter of 31 nm. Nearly all (90%) of the virion particles excluded the negative stain and appeared spherical to polygonal (Fig. 8A). Particles penetrated by the stain appeared to be smaller (30 to 31 nm) and had a more pronounced polygonal appearance (data not shown). All the MBV particles were penetrated by the negative stain and exhibited a pronounced polygonal profile (Fig. 8B). The background of samples of MBV preparations, even after three bandings in CsCl gradients, contained negatively stained extraneous material (data not shown) which was not observed in virion preparations. This material may represent broken MBVs generated during sample preparation for electron microscopy.

with unlabeled HeLa cell rRNAs and centrifuged in 15 to 30% sucrose gradients. Fractions (1 ml) were analyzed for both A260 and acid-precipitable radioactivity. Inset, A 32P-labeled sample of each RNA was also analyzed by electrophoresis in an agarose gel. Arrow shows the position of the RNA.
Negative results that suggest and VP3 VP2, phoresis (Fig. proteins and molecular weights. At various times MBVs each population each time synthesized by plaque in dine and infection, virions B5-infected cultures showed 1) that MBVs were synthesized after each infection. Their yield was maximum when cells were infected with virions alone, minimum in cells infected only with MBVs, and intermediate after infection with a combination of the two. It appears that the MBV population inhibits infection caused by the virion, although the degree of inhibition varied considerably (about 58 to 67%) in three experiments. Furthermore, MBVs can

To ascertain the time of synthesis of MBVs, B5-infected cultures were labeled with [3H]uridine and [35S]methionine. At various times after infection, virions and MBVs were purified by centrifugation in two CsCl gradients and analyzed by plaque assay and polyacrylamide gel electrophoresis. The plaque assay results (Table 1) show that (i) both populations synthesized at each time point were infectious, (ii) the yield of each population increased with time, and (iii) the yield of MBVs was consistently lower. Electrophoresis (Fig. 9) showed that MBVs synthesized at various times contained the same capsid proteins and several additional proteins of various molecular weights. The virions contained VP1, VP2, and VP3 and a trace of noncapsid proteins. Negative results of two pulse-chase experiments suggest that MBVs may not be the progenitor of virion. Experiments are in progress to detect formation of virions from MBVs in cell-free extracts.

Finally, we compared the nature and yield of progeny virions in cells infected with the two populations. Cultures were infected with purified preparations of virions, MBVs, or a combination of the two. At about 20 h p.i., the two populations were purified and plaque assayed. The results (Table 2) showed that both virions and MBVs were synthesized after each infection. Their yield was maximum when cells were infected with virions alone, minimum in cells infected only with MBVs, and intermediate after infection with a combination of the two. It appears that the MBV population inhibits infection caused by the virion, although the degree of inhibition varied considerably (about 58 to 67%) in three experiments. Furthermore, MBVs can

FIG. 5. Comparison of virion RNA (A) and MBV RNA (B) by T1 oligonucleotide fingerprinting. Samples of 32P-labeled virion and MBV RNA were digested exhaustively with RNase T1, and the products were separated by two-dimensional polyacrylamide gel electrophoresis. The three oligonucleotides in the inset of (A) are from another virion RNA preparation.

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FIG. 6. Effects of EDTA on [35S]methionine- and [3H]uridine-labeled B5 MBVs. Equivalent amounts of radioactive virions were treated with 10 mM Tris-hydrochloride (pH 7.4)-10 mM NaCl-1.5 mM MgCl2 (A) or exposed to 20 mM EDTA and analyzed by sedimentation in 15 to 40% linear sucrose gradients (B). The gradient solutions were made for (A) in the above buffer and for (B) in 10 mM Tris-hydrochloride (pH 7.4)-10 mM NaCl-20 mM EDTA. Fractions were collected to determine acid-precipitable radioactivity.
produce infection by themselves without help from the virion.

MBVs are quite stable. Their infectivity did not decrease significantly for 1 to 2 months when the purified virus dialyzed against PBS was stored in the vapor phase of liquid nitrogen.

DISCUSSION

This study shows that group B coxsackie-virus-infected HeLa cells contain a virus-specific, membrane-bound ribonucleoprotein particle not detected previously. These MBV particles have been isolated from a number of freshly harvested cells extracted with Nonidet P-40. They are present in fair amounts in the infected cells, are quite stable, and are infectious, although significantly less so than the virions.

The B5 MBV sediments slower than the virion (at about 107S) and has a lower buoyant density (about 1.30). Its A260:280 of 1.22 suggests the presence of relatively more protein than RNA. Virion and MBV RNA are indistinguishable by velocity sedimentation in sucrose gradients and electrophoresis in denaturing gels, but the T1 oligonucleotide fingerprints appear to be different. MBV RNA resists digestion with RNase, suggesting that this RNA is within and protected by the protein. The protein compositions of virions and MBVs are quite different. MBV contains all but one virion capsid proteins and at least seven additional proteins of various molecular weights. Three of these appear to be of host origin; the rest may be precursors of virion capsid proteins.

<p>| TABLE 1. Plaque assay of virions and MBVs synthesized at various timesa |
|-----------------|-----------------|-----------------|</p>
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<th>Time (h) p.i.</th>
<th>Population</th>
<th>Yield (total PFU × 10⁹)</th>
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<tr>
<td>3.5</td>
<td>Virions</td>
<td>25</td>
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<tr>
<td></td>
<td>MBVs</td>
<td>0.92</td>
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<tr>
<td>5.5</td>
<td>Virions</td>
<td>36</td>
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<td></td>
<td>MBVs</td>
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<td>24</td>
<td>Virions</td>
<td>135</td>
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<td></td>
<td>MBVs</td>
<td>7.6</td>
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a At each time point, about 0.22 × 10⁹ infected (5 PFU per cell) cells were labeled for virus purification by two CsCl gradient centrifugations.
MBVs do not appear to be a population of virions artifactually associated with cytoplasmic membrane proteins during the procedure of virus isolation. Instead, they are a distinct and stable population. Support for this conclusion comes from the following observations.

(i) Electron microscopic analysis of B5 virus shows that MBVs are quite different morphologically from virions. Whereas nearly all (90%) of the virions are intact and impermeable to the negative stain, all of the MBVs are polygonal and penetrated by the stain. Furthermore, the MBVs tend to be smaller, and they have a broader size distribution. In some instances, permeability to stain may indicate a loss of nucleic acid, but in our preparation, it is more likely to reflect a modification of the protein subunits of the coat shell (8). Conformational shifts of shell structure are characteristic of picornaviruses (30).

(ii) The additional proteins of the MBVs are tightly bound to the particle and cannot be dissociated even by repeated CsCl gradient centrifugations. Liberation of the particle requires extraction of infected cells with Nonidet P-40, which disrupts the membrane structure of the cytoplasm.

(iii) Membranes play an active role in picornavirus multiplication. In the case of poliovirus, viral RNA replication and virion formation occur in association with the smooth endoplasmic reticulum (2, 3), and cell-free assembly of 14S particles into 74S empty capsids requires rough membrane fractions (24). The existence of MBVs is consistent with these observations.

Synthesis of virions and MBVs is quite rapid in B5-infected cells. At about 3.5 h p.i., a fair amount of each type could be detected, and synthesis continued for at least 24 h p.i. No differences could be detected among MBVs or virions synthesized at various time points. For reasons as yet unclear, the yield of MBVs is quite variable and always lower than for virions; there is no apparent correlation with the multiplicities of infection.

Infection of cells with MBVs alone produces

<table>
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<th>TABLE 2. Plaque assay of virions and MBVs synthesized in different cultures*</th>
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<td>Infected culture</td>
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</tr>
<tr>
<td>Virions</td>
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<td>MBVs</td>
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<td>Virions + MBVs</td>
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* Cultures of about 0.12 × 10⁹ cells were infected at about 0.2 PFU per cell with gradient-purified virions, MBVs, or a combination of the two and then labeled with [³⁵S]methionine and [³H]uridine for purification of progeny virions by two CsCl gradient centrifugations.
both virions and MBVs, but their yield is less than in cells infected with virions alone. Coinfection with both virions and MBVs produces an intermediate yield, suggesting that MBVs interfere with the production by virions. This interference appears to be more pronounced when the MBVs are adsorbed first (about 40 min earlier), suggesting that the interference occurs at the level of virus adsorption. Further studies are in progress to examine this possibility.

The infectivity of MBVs is not due to contamination with a small amount of virions. MBVs are infectious even after banding in CsCl three times. The data shown in Fig. 1F for B6 and similar data for B5 (not shown) show a total separation of MBVs from virions.

The MBV of this study is similar to L particles of rhinovirus 14 (11) and A particles of rhinovirus 2 (18) in certain features but different in others. MBVs and L particles are lighter, less infectious than virions, and are associated with extraneous material. Unlike rhinovirus 14, however, MBVs can withstand rebanding in CsCl; appear both early and late in infection, and are detectable after labeling in the RNA. MBVs are lighter and contain only three capsid proteins, like A particles. However, unlike A particles, MBVs can attach to host cells. Whether the MBV is a structural intermediate in coinfection remains unknown and is under investigation.

Defective-interfering (DI) particles have been reported in two picornaviruses, poliovirus (6) and mengovirus (23), and in several animal viruses (14, 17). The criteria for DI particles (16, 17) are (i) inability to propagate without helper virus, (ii) a requirement for homologous standard virus as helper for replication, (iii) an ability to decrease the yield of standard virus by interference, and (iv) preferential replication over standard virus. Poliovirus DI particles also show a lower buoyant density than standard virus and contain about 15% less RNA. Mengovirus DI particles contain about 4 to 6% less RNA than standard virus.

MBVs are similar to DI particles in certain features (e.g., lower buoyant density and an ability to decrease the yield of virions by interference), but they are different in others (e.g., MBVs can propagate without helper virus and are associated with several host proteins). It is therefore unclear whether they are true DI particles.

ACKNOWLEDGMENTS

This work was supported in part by grant HRC-11-003 from the New York State Health Research Council, grant HRRI-37035 from the New York State Health Department Biomedical Research Services, and Public Health Service grant GM-27792 from the National Institute of General Medical Sciences.

LITERATURE CITED