〈*Original Article*〉

Analysis of Morphology and Infectivity of Measles Virus Particles

Eriko DAIKOKU, Chizuko MORITA, Takehiro KOHNO and Kouichi SANO

Department of Microbiology, Osaka Medical College, Takatsuki-city, Osaka 569-8686, Japan

Key Words : Measles virus; Infective particle; Morphology; Electron microscopy

ABSTRACT

The measles virus (MeV) shows polymorphisms in morphology and viral particle size, however, the localization of viral proteins and infectivity in viral particles of different sizes have not been well characterized. To determine the localization of viral proteins and the infectivity of viral particles of different sizes, MeV-infected cells and their culture supernatant were analyzed by electron microscopy and membrane filter fractionation. The sizes of MeV-like particles were distributed between 50 and 1000 nm and the major distribution peak was found for particles with diameters of 350-400 nm. The MeV M protein was lined under the envelope of all MeV-like particles and membrane-filter-fractionated MeVs of all sizes showed infectivity. These findings suggest that MeV particles, particularly large particles, can be used as a vaccine by designing a chimera virus containing antigens or genome of other virus species.

INTRODUCTION

Measles is a systemic infectious disease and is spread by droplet nuclei. The measles vaccination effectively prevents a severe outbreak of the disease, but incomplete vaccination in communities can induce an outbreak of measles in children and adults (1). The causative microorganism, the measles virus (MeV), can persistently infect the human brain and induce subacute sclerosing panencephalitis (SSPE) in adolescence (2, 3, 4). For the prevention of MeV transmission and the development of a better MeV vaccine, an analysis of the viral infectivity is important.

Benyesh *et al.* (5) reported that infectious MeV particles are approximately 140 nm in diameter and not polymorphic, by collodion gradocol membrane filtration. Because Benyesh *et al.*'s collodion membrane had the property of adsorbing proteins, they may have lost infective MeV particles as a result of the adsorption of some of the viral particles.

Concerning electron microscopic morphology, MeVs were analyzed in the 1960s (6) and were determined to be polymorphic. The conventional electron micrograph of a MeV particle shows cross, vertical and tangential sections of a nucleocapsid and an envelope with surface projections. Nakai and Imagawa (7) reported that the sizes of MeV particles range between 180 and 600 nm, and Lund *et al.* (8) and Nakai *et al.* (9) reported particles with diameters of 300-1000 nm. The nucleocapsid was also morphologically studied in MeV and defective MeV from SSPE patients (10). Although in those studies, the size

Address correspondence to:
Kouichi Sano M.D., Ph. D., Department of Microbiology and Infection Control, Osaka Medical College,
2-7 Daigaku-machi, Takatsuki-city, Osaka 569-8686, Japan
Phone: +81-72-684-7367 Fax: +81-72-684-6517 E-mail: ksano@art.osaka-med.ac.jp

of MeV was determined by detecting the envelope, surface projections and nucleocapsid in particles, the infectivity of these particles is still unknown.

Miller and Raine (11) reported that MeV particles are partially separable into three fractions by sucrose density ultracentrifugation. The fractions are all infectious and contain particles 770 ± 270 nm, 465 ± 110 nm and 410 ± 120 nm in size. They also demonstrated that all three fractions contained proteins that showed the same molecular weight as MeV components, but they did not analyze which sized particle has antigen and infectivity. Vincent *et al*. (12) analyzed the localizations of MeV H and N proteins using a pre-embedding immunoelectron microscopic technique; however, they did not study the infectivity of MeV.

The determination of infectious MeV particles is important in considering a carrier of a chimeric vaccine. It has not yet been determined which MeV particle size is infective at present because appropriate methodologies are not available. Recent advances in immunoelectron microscopy for microbiology (13, 14) have facilitated the determination of the Borna disease virus particle (15) and the discovery of the intrabacterial nanotransportation system for bacterial enzyme (16) and cytotoxin (17). In addition, commercially available membrane filters have become efficient and may enable fractionation at the size level of MeV particles.

In order to determine which MeV particle size is infective, MeV particles are studied by immunoelectron microscopy and fractionation with membrane filters.

MATERIALS and METHODS

Virus culture and harvest: MeV, the Edmonston strain, was inoculated into African green monkey kidney cells, Vero cell line, and cultured in a minimum essential medium containing 1% fetal bovine serum at 37 ℃ for 7 days with medium changing. The supernatant of the culture was harvested, clarified by centrifugation at 3000 rpm for 15 min, passed through a 3-μm-pore-size membrane filter (MF-Millipore™ membrane filter, Millipore, Japan) and stored at –80 ℃ until use. The infected cells were harvested and used as an electron microscopic sample.

Conventional electron microscopy: To confirm whether our MeV produces polymorphic particles of various sizes as previously described, the MeV-infected cells were observed by conventional electron microscopy. The MeV-infected cells were fixed with 0.2% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.2) for 3 min at room temperature and harvested with a plastic scraper. The cells were suspended in 2% glutaraldehyde in the same buffer at 4 ℃ for 60 min and washed 5 times in the buffer. The fixed cells were further fixed in 1% osmium tetraoxide in the same buffer at 4 ℃ for 60 min, washed in the buffer, dehydrated in a graded ethanol series and embedded in Epon 812 resin. Ultrathin sections were made using an ultramicrotome (Reichert ULTRACUT N, Reichert, Austria), double stained with uranyl acetate and lead citrate, and observed under an electron microscope (H-7100, Hitachi, Japan) with an accelerating voltage of 100 kV. The particles with a nucleocapsid-like structure inside or a projection-like structure on the surface were determined to be MeV-like particles.

Furthermore, to determine the distribution of the diameters of MeV-like particles, the number of MeV-like particles was counted for 50 nm ranges in diameter, and plotted on a graph.

Negative staining in electron microscopy: The MeV-like particles in the ultrathin section may have been part of cell structures, such as cellular villi and swelling. To confirm whether the MeV-like particles were solitary particles, purified virus-like particles were examined by negative staining. The culture supernatant was purified by sucrose step gradient ultracentrifugation, and each fraction was resuspended in phosphate buffer saline (PBS) and washed by ultracentrifugation. The pelleted virus was resuspended in distilled water and a drop of the resuspension was mounted on an ion-coated copper grid supported by a carbon-coated collodion film, and fixed with 0.5% glutaraldehyde in PBS for 2 min. The grid was stained with 1% uranyl acetate for 2 min and observed under an electron microscope (H-7100). Particles containing a nucleocapsid inside or a projection-like structure on the surface were determined to be MeV-like particles.

Size fractionation and virus titration: To determine the infectivity of viral particles of different sizes, the culture supernatant of viral infected cells was passed through membrane filters with pore sizes of 0.8, 0.45 and 0.22 μm. Each filtrate was serially diluted and inoculated into Vero cells cultured in a 96-well microplate. After 7 days of culture, the cells in the microplate were fixed and stained with Giemsa solution, and observed under a converted microscope; the cytopathic effect (CPE) was detected. The

$TCID_{50}$ (50% tissue culture infective dose) was calculated using the method of Reed and Muen (18).

Immunoelectron microscopy: To determine whether the MeV-like particles contain the MeV antigen, the particles budding from virus-infected cells were examined by immunoelectron microscopy. MeV-infected cells were fixed with 0.2% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.2) for 3 min at room temperature and harvested with a plastic scraper. The collected cells were fixed again with 1% glutaraldehyde in 0.05 M cacodylate buffer at 4 ℃ for 60 min, dehydrated in a graded ethanol series, and embedded in Lowicryl K4M resin. Ultrathin sections were made in the same manner as for conventional electron microscopy and mounted on a nickel grid supported by a carbon-coated collodion film. The ultrathin sections on the grid were treated with 5% goat serum in 0.15 M PBS (pH 7.2) to block nonspecific reactions. The sections were reacted with a drop of mouse antiserum against MeV M and H proteins (Chemicon, CA, USA) at room temperature for 60 min. After three times washing, the sections were then reacted with goat serum containing 10 nm colloidal gold-labeled anti-mouse IgG (Amersham, England) at room temperature for 60 min and washed in the PBS. The immunostained sections were fixed with 1% glutaraldehyde in 0.05 M cacodylate buffer and washed in distilled water. The immunolabeled sections were treated with a mixture of 0.01% ruthenium red and 0.5% osmium tetraoxide in 0.05 M cacodylate buffer at room temperature for 10 min, and double stained with uranyl acetate for 20 min and lead citrate for 1 min, as previously described (13). The sections were observed under an electron microscope.

RESULTS

The MeV-like particles budded from infected cells that had an inclusion body in their cytoplasm (**Fig. 1A**). The shape of each particle was mainly round (**Figs. 1A-E**), but occasionally tadpole shaped (**Fig. 1A arrows**), rod-like (**Figs.1B arrow and F**) and gourd-like (**Figs. 1G, H and I**). The particles were found to be polymorphic and had sizes between 50 nm and 1000 nm diameter. In particular, the envelope of the large particles was thinner than the envelope of small particles (**Figs. 1J, K**).

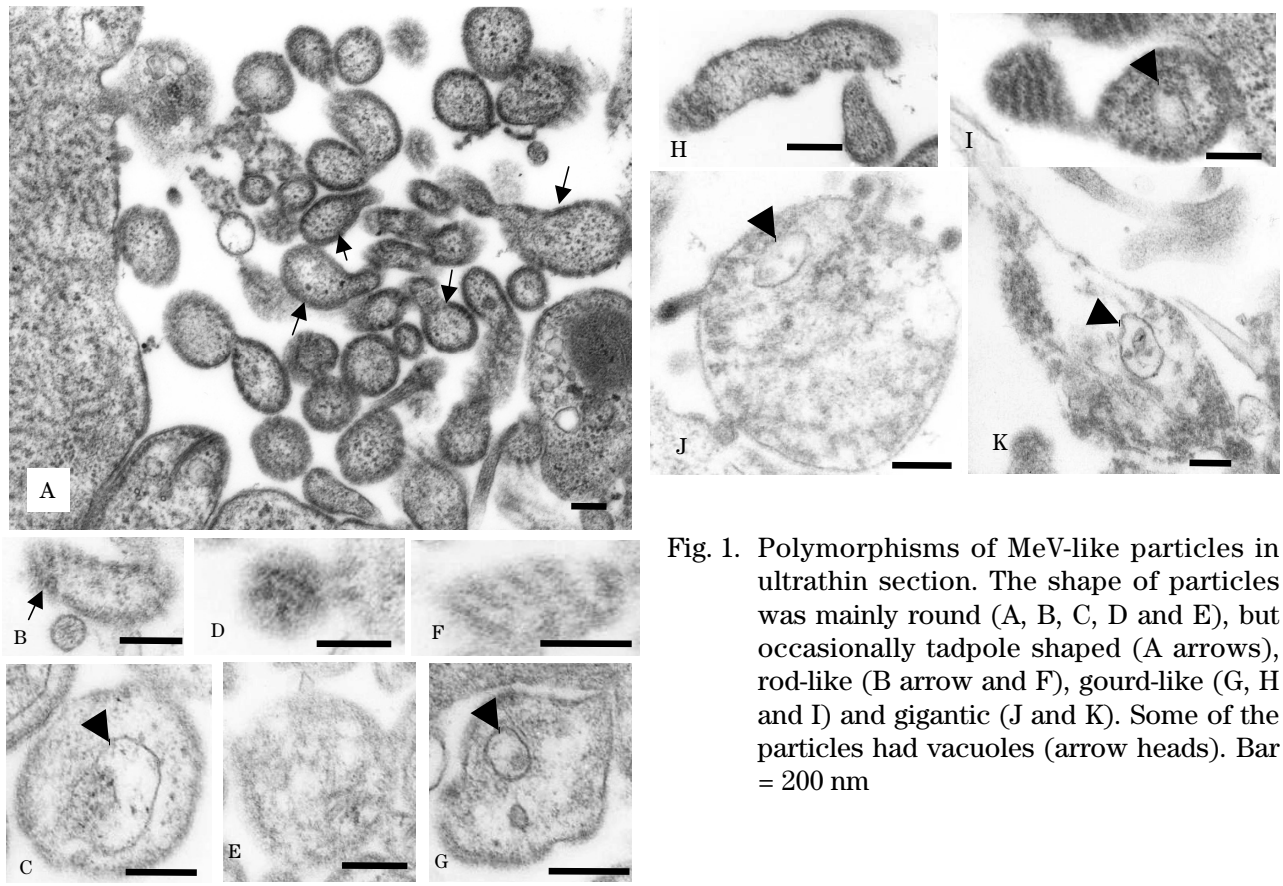

Fig. 1. Polymorphisms of MeV-like particles in ultrathin section. The shape of particles was mainly round (A, B, C, D and E), but occasionally tadpole shaped (A arrows), rod-like (B arrow and F), gourd-like (G, H and I) and gigantic (J and K). Some of the particles had vacuoles (arrow heads). Bar = 200 nm

In the distribution of the diameters of MeV-like particles, the major distribution peak was observed for particles with diameters of 350-400 nm (**Fig. 2**). Other small peaks in the range of 150-200 nm, 500-550 nm and 700-750 nm were detected.

Representative electron micrographs of MeV-like particles that corresponded to each peak on the distribution graph are shown in order of their size (**Figs. 3A-E**). The smaller MeV-like particles revealed projections on their surfaces (**Fig. 3A**). MeV-like particles sized 350-400 nm each showed a projection and a nucleocapsid-like structure (**Fig. 3B**). Other larger particles showed nucleocapsid-like structures inside the particle (**Figs. 3C-E**).

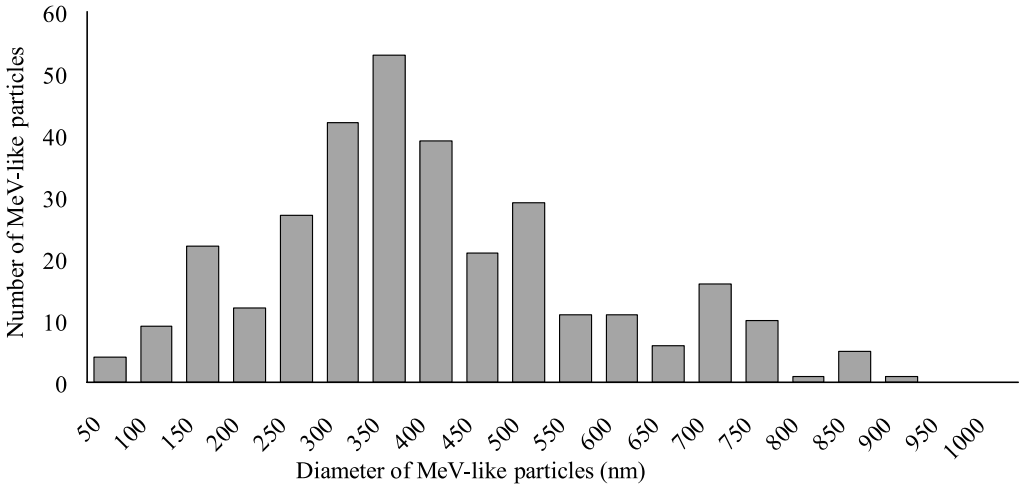

Fig. 2. Distribution of MeV-like particles. There is a major peak at 350-400nm. Three minor peaks at 150-200 nm, 500-550 nm and 700-750 nm are also observed.

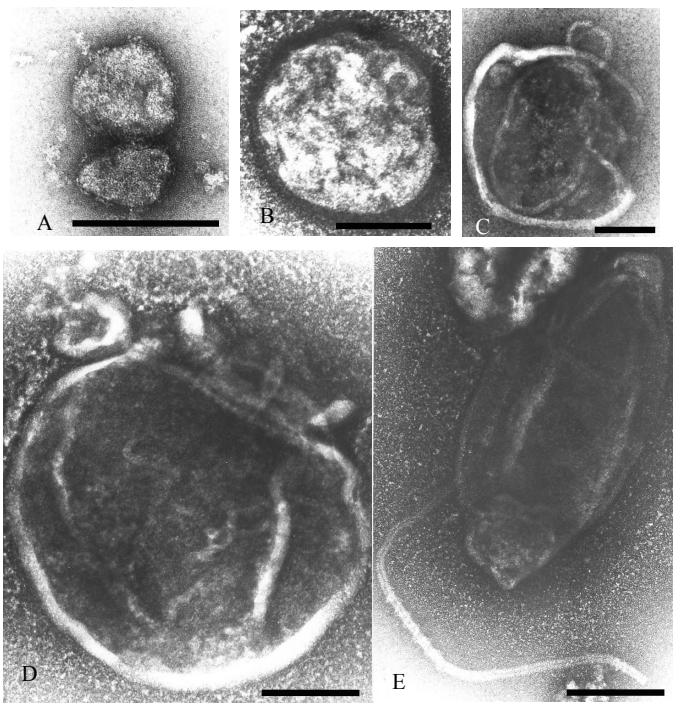

Fig. 3. Polymorphisms of MeV-like particles by negative staining. The shape of smaller particles is intact and spherical (A and B) but larger particles seem to be punctured and the nucleocapsid protrudes to the outside (C, D and E). Bar = 200 nm

Immunoreactivity of anti MeV M protein that locate inner surface of membrane and serve virion assembly and budding are shown in **Figs. 4A-E**. The reactivity of antibodies on MeV-like particles budding from infected cells (**Fig. 4A**) and on an inclusion body in the cytoplasm (**Fig. 4A insert**) was specific. MeV-like particles with sizes smaller than 550 nm showed a clear lining of the M protein inside of the envelope (**Figs. 4B, C, D**); however, larger particles and the thin envelope particles did not (**Fig. 4E**). On the other hand, the H protein, which is a spike and receptor-binding protein, was detected on viral particles in the same manner as the M protein. However, for MeV-like particles of all sizes, the H protein was scattered inside the envelope, unlike the M protein (**Figs. 4F, G, H, I**).

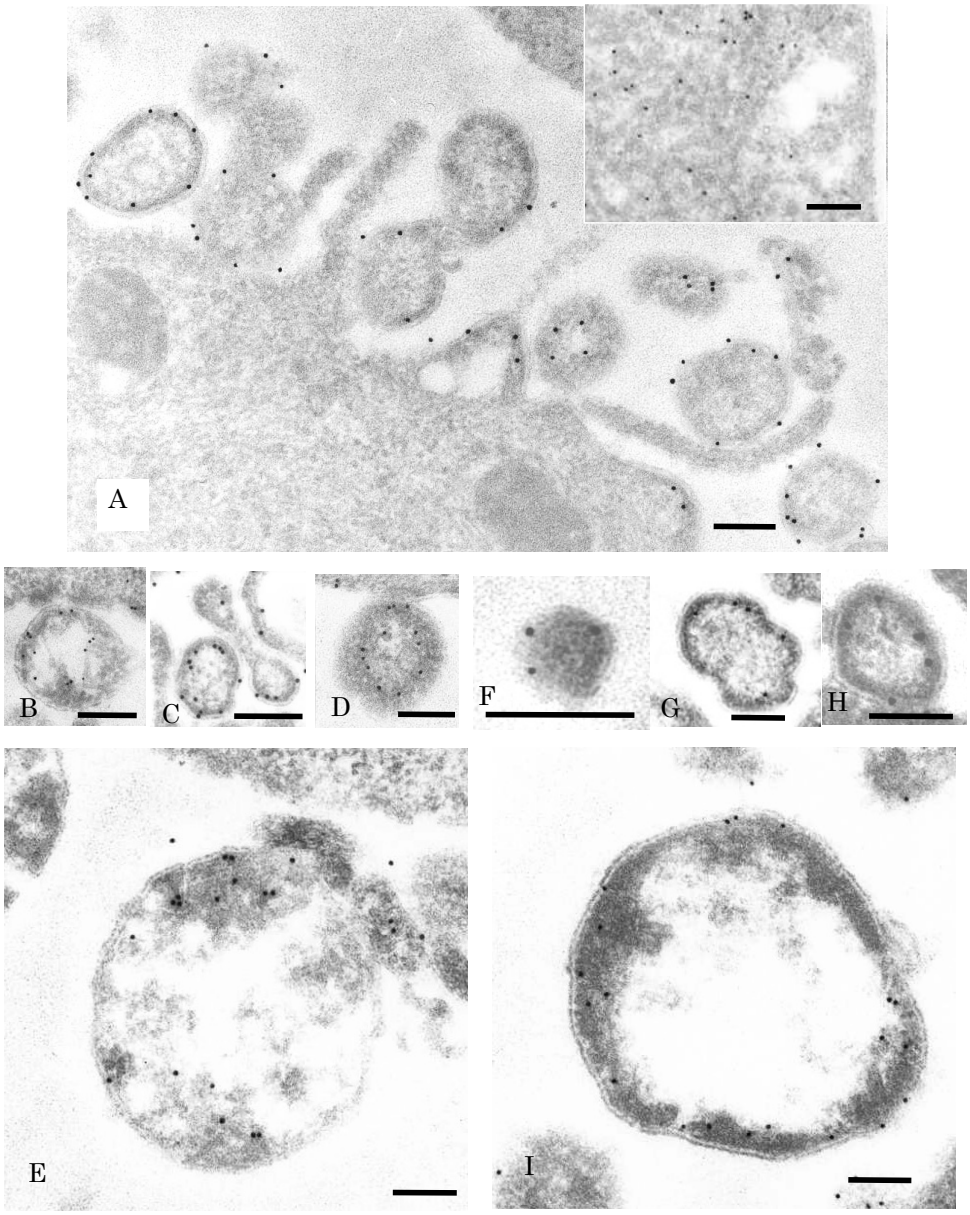

Fig. 4. Immunoelectron micrographs of MeV-like particles. An anti-measles M monoclonal antibody (A to E) and an anti-measles H monoclonal antibody (F to I) were used to reveal pleiomorphic MeV particles. Bar = 200 nm

All the filtrates that were passed through membrane filters with pore sizes of 3.0, 0.8, 0.45 and 0.22 μm exhibited infectivity. The infective titer of the supernatant passed through a 3.0-μm-pore-size filter was 4.1 log $TCID_{50}$/0.1ml, and the titer was reduced to 3.0 log $TCID_{50}$/0.1ml by passing the supernatant through a 0.8-μm-pore-size filter, to 1.5 log $TCID_{50}$/0.1ml by passing it through a 0.45-μm-pore-size filter, and to 1.2 log $TCID_{50}$/0.1ml by passing it through a 0.22-μm-pore-size filter (**Fig. 5**).

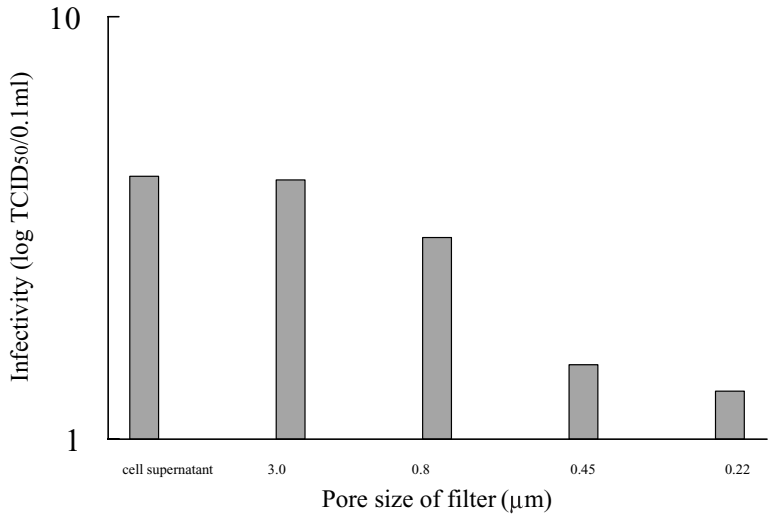

Fig. 5. Infectivity of MeV particles. As the pore size of the membrane filters decreases, the infective titer of the MeV filtrate decreases.

DISCUSSION

In this study, the sizes of MeV particles were analyzed by ultrathin sectioning and negative staining. Since ultrathin sectioning provides more virus-like particles than negative staining does, we observed virus-infected cells by the sectioning methods to estimate the possible size of the MeV particle. Because the images of ultrathin sectioning show sections of particles, the particle images do not always indicate the maximum diameter, and a particle-like structure may be the result of a bleb from cells. After estimating the range of particle diameter, we used negative staining to determine the existence of virus-like particles corresponding to the particles of the same size in the observation of ultrathin sections. By combining the two methods, we confirmed that large MeV-like particles are solitary particles, not blebs from host cells, and that MeV particles show a wider range of diameters than that previously reported (7, 8, 9).

Since we have found that MeV particles mainly sized of 350-400 nm in diameter, the size may be most stable for virus morphogenesis. Lund *et al* (8) suggested that the number of nucleocapsids is a determinating factor of size. In the present study, MeV particles sized smaller than 220 nm revealed infectivity suggesting that the smaller particle is complete virus particle, and that larger sized particle possesses two or more nucleocapsids. Virological and biological significances of different sized MeV particles could not be clarified in this study.

Recently, a chimeric vaccine has been constructed using a molecular biological technique, and relatively large microorganisms, such as the vaccinia virus (19) and the *Mycobacterium bovis* BCG strain (20). The diameter of the MeV particle depends on the number of nucleocapsids inside the envelope (8), indicating that MeV may have the capacity to contain a large number of nucleocapsids of other viruses. In this study, to clarify whether a large MeV could be applicable as a carrier of the chimeric vaccine, we focused on large MeV particles.

Because post-embedding immunoelectron microscopy for viruses was not originally designed for virus morphology, almost no study of MeV morphology has been performed. Vincent *et al*. (12) reported on a morphological analysis of MeV

antigen location; however, they applied pre-embedding immunoelectron microscopy mainly for the observation of the viral surface antigen. Post-embedding immunoelectron microscopy is useful for analyzing antigen localization within an envelope. By using our post-embedding immunoelectron microscopic technique (13), a packaging protein (M protein) and a receptor-binding protein for infection (H protein) were detected in large MeV-like particles, as well as in small ones in this study.

Because large MeV-like particles possess the MeV M and H proteins and a nucleocapsid, they are considered to be complete virus particles. To confirm the infectivity of these large virus particles, we further examined the infectivity of those particles fractionated by membrane filters. The results indicated that the virus particles with sizes between 3.0 and 0.8 μm, 0.8 and 0.45 μm, and 0.45 and 0.22 μm, and less than 0.22 μm exhibit infectivity. Particularly, since the filtration of virus suspensions through an 0.8 μm membrane filter reduced their infectivity, MeV particles larger than 0.8 μm are considered to be infectious in addition to those with diameters less than 0.8 μm. Filtrate that was passed through a 0.22-μm-pore-size membrane filter showed infectivity, and MeV particles of approximately 0.2 μm in size must possess at least one complete nucleocapsid.

A chimeric virus of MeV and the Human immunodeficiency virus (HIV) has been constructed successfully (21). A practical consideration of vaccination leads us to the need to develop a chimeric virus of MeV and the rubella virus (RV). To probe whether large MeV particles are suitable for packaging RV nucleic acids, we attempted a superinfection of both MeV and RV to create chimeric viruses. Both viral proteins were detected in cells dually infected with both viruses (data not shown). These findings may indicate that a MeV-RV chimera virus was possibly constructed.

In conclusion, MeV particles are polymorphic and have a diameter ranging between 50 and 1000 nm, and particles of all sizes are infective. These results suggest that MeV, particularly large particles, can be a possible carrier of foreign viral antigens or the genome for a chimeric vaccine.

ACKNOWLEDGEMENTS

We thank Dr. T. Nakano, Dr. W. Hong and Mr. Y. Fujioka for helpful advice

REFERENCES

1. Takayama N. Measles and rubella. Rinsho Byori. 2005;53:845-52.(in Japanese)
2. Murray PR, Baron EJ, Jorgensen JH, Pfaller MA, Yolken RH. Manual of clinical microbiology, 8th ed. Washington D.C: ASM Press, 2006:1389-90pp.
3. Hall WW, Choppin PW. Evidence for lack of synthesis of the M polypeptide of measles virus in brain cells in subacute sclerosing panencephalitis. Virol 1979;99:443-7.
4. Rozenblatt S, Gorecki M, Shure H, Prives CL. Characterization of measles virus-specific proteins synthesized in vivo and in vitro from acutely and persistently infected cells. J Virol 1979;29:1099-106.
5. Benyesh M, Pollard EC, Opton EM, Black FL, Bellamy WD, Melnick JL. Size and structure of echo, poliomyelitis, and measles viruses determinated by lonizing radiation and ultrafiltration. Virol 1958;5:256-74.
6. Waterson AP, Cruickshank JG. The nature of measles virus. Virol 1961;15:379-82.
7. Nakai M, Imagawa DT. Electron microscopy of measles virus reprication. J Virol 1969;3:187-97.
8. Lund GA, Tyrrell DLJ, Bradley RD, Scraba DG. The molecular length of measles virus RNA and structural organization of measles nucleocapsids. J gen Virol 1984;65:1535-42.
9. Nakai T, Shand FL, Howatson AF. Development of measles virus iv vitro. Virol 1969;38:50-67.
10. Nakajima H. Electron microscopic observations on the nucleocapsids both in the cells infected with measles virus and in subacute sclerosing panencephalitis (SSPE) latent infection cells. Bulletin of the Osaka Medical College. 1982;41:1-14.(in Japanese)
11. Miller CA, Raine CS. Heterogeneity of virus particles in measles virus. J gen Virol 1979;45: 441-53.
12. Vincent S, Spehner D, Maniè S, Delorme R, Drillien R, Gerlier D. Inefficient measles virus budding in murine L.CD46 fibroblasts. Virol 1999;265:185-95.
13. Kohno T, Fujioka Y, Goto T, Morimatsu S, Morita C, Nakano T, Sano K. Contrast-enhancement for the image of human immunodeficiency virus from ultrathin section by immunoelectron microscopy. J Virol Methods 1998;72:137-43.
14. Hong W, Morimatsu S, Goto T, Sachs G, Scott

DR, Week DL, Kohno T, Morita C, Nakano T, Fujioka Y, Sano K. Contrast-enhanced immunoelectron microscopy for *Helicobacter Pylori*. J Microbiol Methods 2000;42:121-7.
15. Kohno T, Goto T, Takasaki T, Morita C, Nakaya T, Ikuta K, Kurane I, Sano K, Nakai M. Fine structure and morphogenesis of Borna disease virus. J Virol 1999;73:760-6.
16. Hong W, Sano K, Morimatsu S, Scott DR, Weeks DL, Sachs G, Goto T, Mohan S, harada F, Nakajima N, Nakano T. Medium pH-dependent redistribution of the urease of *Helicobacter Pylori*. J Med Microbiol 2003;52:211-6.
17. Hong W, Nakano T, Daikoku E, Morita C, Kohno T, Lian HH, Sano K. Intrabacterial proton-dependent CagA transport system in *Helicobacter Pylori*. J Med Microbiol 2005;54:1117-25.
18. Reed LJ, Muench H. A simple method of estimating fifty per cent endpoints. Am J Hygene 1938;27:493-7.
19. Moss B. Genetically engineered poxviruses for recombinant gene expression, vaccination, and safety. Proc Natl Acad Sci USA 1996;93:11341-8.
20. Honda M, Matsuo K, Nakasone T, Okamoto Y, Yoshizaki H, Kitamura K, sugiura W, Watanabe K, Fukushima Y, Haga S, Kastura Y, Tasaka H, Komuro K, Yamada T, Asano T, Yamazaki A, Yamazaki S. Protective immune responses induced by secretion of a chimeric soluble protein from a recombinant *Mycobacterium bovis* bacillus Calmette-Guérin vector candidate vaccine for human immunodeficiency virus type 1 in small animals. Proc Natl Acad Sci USA 1995;92:10693-7.
21. Lorin C, Mollet L, Delebecque F, Combredet C, Hurtrel B, Charneau P, Brahic M, Tangy F. A single infection of recombinant measles virus vaccines expressing human immunodeficiency virus (HIV) type 1 clade B envelope glycoproteins induces neutralizing antibodies and cellular immune responses to HIV. J Virol 2004;78:146-57.

Received November 22, 2006
Accepted December 21, 2006